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CELL-MEDIATED IMMUNITY IN VIVO AND IN VITRO BY CHICKENS WITH BCG OR MAREK'S DISEASE INFECTION AND THE EFFECTS OF BURSECTOMY AND THYMECTOMY ON THE EXPRESSION OF CELL-MEDIATED IMMUNITY

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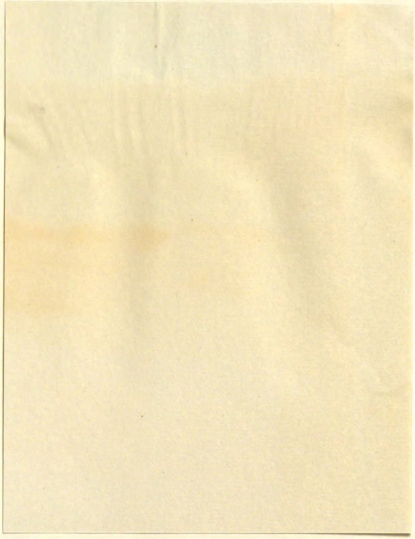

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ABSTRACT

CELL-MEDIATED IMMUNITY IN VIVO AND IN VITRO BY CHICKENS WITH BCG OR MAREK'S DISEASE INFECTION AND THE EFFECTS OF BURSECTOMY AND THYMECTOMY ON THE EXPRESSION OF CELL-MEDIATED IMMUNITY

By

Inguna Silavs Fauser

A method was developed to detect the migration inhibition factor (MIF), an in vitro correlate of delayed sensitivity, from peripheral leucocytes of chickens inoculated with Bacille Calmette-Guerin (BCG) or Marek's disease virus (MDV).

There was no radial migration of the blood leucocytes from spots on a plastic petri dish in the presence of a tuberculo-protein (B-24) or A-antigen of Marek's disease (MD). Leucocytes from chickens sensitized with BCG or MDV were inhibited in in vitro migration by B-24 and A-antigen respectively. MIF was not detected by B-24 in complete Freund's adjuvant (CFA) inoculated chickens.

B-24 elicited more delayed skin reactions in BCG than CFA sensitized groups: eight of eight as compared to five of eight. Skin reactions to old tuberculin (OT) were positive in six of eight CFA inoculated chickens. Both OT and B-24 elicited delayed skin reactions in five of eight CFA

inoculated chickens. All eight CFA and seven of eight BCG inoculated chickens had granulomatous lesions and/or acid fast organisms at the site of inoculation and all inoculated chickens had precipitating antibody with purified protein derivative (PPD).

A-antigen elicited delayed skin reactions in all adult chickens infected with MDV. None of the five chickens inoculated with MDV in CFA had delayed skin reactions with A-antigen but all did with OT. MDV infected chickens had fluorescent antibody to MDV infected fibroblasts but no gross or microscopic MD lesions were detected.

Three groups of chickens were used to determine if neonatal thymectomy would eliminate MIF production. Intact, bursectomized, and thymectomized chickens were negative on two tests three weeks apart for MIF production to B-24 prior to inoculation with BCG. They were also negative for precipitating antibody to PPD and delayed skin reactions to B-24. The tests were repeated after sufficient time for immune responses to have developed. Intact chickens had detectable antibody, MIF, and delayed skin reactions. All thymectomized chickens had antibody, three of seven had delayed skin reactions with no Arthus responses to B-24, and only one of seven had detectable MIF. Only the bursectomized chickens had true delayed skin reactions to OT.

Three of seven intact and six of seven thymectomized chickens had Arthus reactions with OT.

In vivo graft-versus-host (GVH) reactions by blood leucocytes of chickens were greater by leucocytes from thymectomized donors than from the intact or bursectomized chickens.

Detection of MIF in avian blood leucocytes is reproducible and correlates with delayed skin reactions. The results support evidence of the thymic role in the capacity to develop delayed sensitivity and MIF production, and indicate that the GVH reactivity by blood leucocytes may not be by the same population of thymic dependent cells as those capable of recruitment for MIF production.

CELL-MEDIATED IMMUNITY IN VIVO AND IN VITRO BY CHICKENS WITH BCG OR MAREK'S DISEASE INFECTION AND THE EFFECTS OF BURSECTOMY AND THYMECTOMY ON THE EXPRESSION OF CELL-MEDIATED IMMUNITY

By

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DEDICATION

To Dr. Anne VanderWoude Miller, Instructor in the Life Science Department and Dr. Marinus Swets, Chairman of the English Department, Grand Rapids Junior College, Grand Rapids, Michigan, in appreciation of their talents as teachers.

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INTRODUCTION

The adaptive immunity of vertebrates includes the ability to produce immunoglobulins and to develop cellular immunity in response to antigenic stimulation. Cellular immunity which includes delayed sensitivity is mediated by specifically sensitized lymphocytes. It is responsible for homograft rejection, graft-versus-host reactivity and immunity to various microorganisms. It can not be transferred with serum, only by lymphoid cells or their extracts.

The tests for varied functions of the sensitive lymphocyte in vitro were recently developed and are referred to as in vitro correlates of delayed sensitivity. Because both immune systems, immunoglobulins and sensitive lymphocytes, can now be assayed in vitro, there has been an intensive search for the mechanism(s) which control(s) the development and function of adaptive immunity. Thereby many untreatable diseases including neoplasms and autoimmunity may be treated by the appropriate "immunologic engineering", i.e., replacement of inadequate or defective immunologic function or suppression of immunologic responses.

The chicken as a representative of the class Aves is a unique experimental laboratory model of adaptive immunity

because two anatomically separated central lymphoid organs control the development of immunologic competence. The bursa of Fabricius, a hindgut lymphoid organ, is responsible for the system of immunoglobulin production which is comprised of lymphocytes of the germinal centers and plasma cells of the peripheral lymphoid tissue. The other central lymphoid organ, the thymus, is responsible for the development of the thymus-dependent small lymphocytes of delayed sensitivity. The anatomical discreteness of the two central lymphoid organs in the chicken facilitates the selective study of either the immunoglobulin or cell-mediated immunologic system. Removal of either the bursa or thymus early in life compromises or eliminates the development of the respective immunologic capacity.

This is a report of the results of research to investigate the following questions: (1) Is Migration Inhibition Factor (MIF), an in vitro correlate of delayed sensitivity, one of the biological effector molecules elaborated when sensitive lymphocytes from chickens are incubated in vitro with antigen? (2) Does Marek's disease, a lymphoproliferative disease in chickens, evoke delayed sensitivity to a soluble antigen of the Marek's disease virus (MDV)? (3) Does MIF production correlate with in vivo expressions of cellular hypersensitivity? (4) Does neonatal thymectomy results in compromised in vivo delayed sensitivity and in vitro MIF production as predicted?

LITERATURE REVIEW

Dependence of Immunologic Development of the Chicken on the Thymus and Bursa of Fabricius

The development of adaptive immunity in the chicken is dependent upon the two anatomically separated central lymphoid organs, the thymus comprised of 5-7 lobes on each side of the cervical vertebrae, and the bursa of Fabricius, a single hindgut lymphoid organ. A functional dissociation of the immune response in the chicken was first postulated by Szenberg and Warner (1962) and Warner et al. (1962), and confirmed by Aspinall et al. (1963), Jankovic and Išvaneski (1963), Cooper et al. (1965), and Cain et al. (1968).

The thymus in chickens and mammals, and thymus-dependent small lymphocytes (T cells, T lymphocytes), are responsible for the development of delayed type hypersensitivity or cellular immunity (Miller, 1961; Lawrence and Landy, 1969). This includes immunologic surveillance as exemplified by homograft rejection and graft-versus-host reactivity, reactions induced by intracellular microbial chronic diseases with persistent delayed hypersensitivity skin reactions, and development of the untoward reactions of autoimmune diseases. It may also be involved in antigen

recognition or other "helper" function in the humoral or antibody mediated system.

The bursa of Fabricius is largely responsible for the capacity to develop humoral immunity (Chang et al., 1955; Glick et al., 1956). Humoral immunity is mediated by antibodies, the immunoglobulins which are complex protein globulins synthesized by plasma cells. The appendix of the rabbit is thought to function as a central lymphoid organ analogous to the avian bursa (Konda and Harris, 1966). The equivalent structure in other mammals has not been resolved, but it is generally considered to be some gut-associated tissue such as Peyer's patches, tonsils, and/or the appendix (McKneally and Good, 1971; Perey and Guttman, 1972).

Van Alten and Menwissen in 1972 reported antibody production by bursal lymphocytes after sheep erythrocytes were injected into the bursal lumen. There is little evidence to indicate that the lymphoid cells within the bursa itself routinely produce specific antibody directed against a wide variety of experimentally injected antigenic substances (Dent and Good, 1965; Abramoff and Brien, 1968a; Choi and Good, 1973). Abramoff and Brien (1968a) reported marked cell differentiation occurred in the bursa after intravenous immunization with sheep erythrocytes. Bursal lymphocytes (B cells, B lymphocytes) do produce immunoglobulins but have not always been characterized as reacting in some

detectable way with the sensitizing antigen (Marinkovich and Baluda, 1966; Glick and Whatley, 1967; Thorbecke et al., 1968; Kincade and Cooper, 1971; Bankhurst et al., 1972). Nonetheless, higher levels of immunoglobulin bearing cells occur in the bursa than any other lymphoid organ in the chicken (McArthur et al., 1971; Rabellino et al., 1971), or in any mammalian lymphoid organ (Takahashi et al., 1971). Restoration of the capacity for immunoglobulin production follows transplantation of B cells into agammaglobulinemic recipients (Cooper et al., 1966b; Gilmour et al., 1970). It has been suggested that the pool size of B lymphocytes detected by surface immunoglobulins in bursa-dependent lymphoid areas is relatively fixed and dependent on the number seeded initially from the bursa and this is dependent upon the time allowed for the bursa to function before its removal (Kincade and Cooper, 1971; Kincade et al., 1973).

Immunoglobulins are released and distributed into many fluids and secretions including serum (Leslie and Clem, 1969; Lebacq-Verheyden et al., 1972; Bienenstock et al., 1972), bile (Bienenstock et al., 1972; Lebacq-Verheyden et al., 1972; Leslie and Martin, 1973), bronchiorespiratory secretions (Leslie and Martin, 1973), saliva and oviduct washings (Orlans and Rose, 1972), extracts of the chicken caeca (Orlans and Rose, 1972) and the egg (Kramer and Cho, 1970). To date, three classes of immunoglobulins (Ig) have

been identified in the chicken, IgM (Leslie and Clem, 1969) IgY or IgG (Leslie and Clem, 1969), and IgA (Bienenstock et al., 1972; Lebacq-Verheyden et al., 1972; Orlans and Rose, 1972).

The small B lymphocytes proliferate and differentiate into plasma cells. Plasma cells can be identified in vitro by immunofluorescence (Rabellino et al., 1971). Using this method, IgM production has been detected in the bursa as early as 14 days embryonation, and IgG production around the time of hatching (Kincade and Cooper, 1971; Cooper et al., 1972). It has not been determined whether these immunoglobulins are formed in response to antigenic stimuli. They are also present in embryonating chicks obtained from dams raised gnotobiotically (Thorbecke et al., 1968) and 8 day-old chicks reared under a germ-free environment had IgM on the bursa and spleen cells similar to controls. IgA is not detectable in the serum until chickens are several months old (Martin and Leslie, 1973). IgA production is thought to require "helper" function from thymus-dependent lymphocytes. IgA is found in high concentrations in most exocrine secretions such as bile (Orlans and Rose, 1972).

Structure and Development of the Thymus

In the adult chicken, the thymus consists of a variable number of paired lobes which extend from the anterior

cervical to the anterior thoracic regions adjacent to the jugular veins (Janković and Isaković, 1964; Warner, 1964; Lucas and Stettenheim, 1965; Druet and Janigan, 1966). The posterior lobes are intimately associated with the thyroid, parathyroid, and ultimobranchial bodies; sometimes, the capsule surrounding the posterior lobe is absent and lymphoid nodules penetrate the thyroid and parathyroid tissue (Payne and Breneman, 1952; Fauser, 1969; Panigrahi, 1970; Panigrahi et al., 1971; Payne, 1971). The thymus is reported to be the first lymphoid organ to begin development in the chick embryo. This commences around 5 days embryonation with outgrowths of the ventral and lateral walls of the third and fourth pharyngeal pouches with possible contribution from branchial ectoderm (Venzke, 1952; Hammond, 1954).

The origin of thymic lymphocytes (thymocytes) in all vertebrates reported is controversial. Originally two theories were proposed, neither mutually exclusive: The first proposed that epithelial cells within the developing mouse thymus transformed into thymocytes (Auerbach, 1961 as summarized in 1964) and the second theory stated that thymocytes were derived from extrinsic mesenchymal cells (stem cells) which migrated into the epithelial anlage, and then differentiated into thymocytes (Kingsbury, 1940). The second theory, on the basis of parabiotic studies of chick embryos of the opposite sex, is gaining acceptance (Moore and Owen,

1967). Large basophilic cells, derived from the embryo of the opposite sex, were found in the mesenchyme and blood vessels around the thymus at 8 and 9 days of embryonation.

Owen and Ritter reported in 1969 that stem cells first enter the thymic anlage between 6 and 7 days incubation, and give rise to the basophilic cells, which are the precursors of thymic lymphocytes, between 8 and 9 days of incubation. These cells are presumably derived from the yolk sac, as determined by grafting diffusion chambers containing embryonic thymus on the chorioallantois of 9 day chick embryos, and appear to differentiate into thymocytes under the inductive influence of thymic epithelium (Owen and Ritter, 1969). Lucas and Jamroz (1961) reported that lymphocytes are found in the thymus around 11 days embryonation, and by 13 days embryonation cortical and medullary zones are apparent in the thymus. Papermaster and Good (1962) reported large lymphoid cells at 14 days embryonation. The medulla of the thymus contains thymocytes and epithelial cells forming Hassalls' corpuscles, and the cortex is comprised of densely packed small thymocytes in a supportive network of reticular cells (Peterson and Good, 1965; Payne, 1971).

The bone marrow is thought to serve as the source of thymic stem cells in the adult mouse (Moore and Owen, 1966; McGregor et al., 1971) and probably in other mammals as well.

Relationship of the Thymus to Other Lymphoid Organs

Thymus-dependent lymphocytes in mammals and chickens are established peripherally by a mechanism unresolved. A humoral factor of thymic origin may affect cells in the peripheral lymphoid tissues (Osoba and Miller, 1963), or thymocytes may migrate to peripheral areas (Nossal and Goorie, 1964; Murray and Woods, 1964; Woods and Linna, 1965; Larsson, 1966; Törö and Oláh, 1967; Owen and Ritter, 1969; Sainte-Marie and Peng, 1971). In the chicken, thymus-dependent small lymphocytes are located in the blood (Warner et al., 1962; Janković and Isaković, 1964; Isaković and Janković, 1964; Jaffe, 1966; Fauser, 1969; Fauser et al., 1969; Longenecker and Breitenbach, 1969; Fauser et al., 1973b), in the periarteriolar zone comprising the white pulp of the spleen (Janković and Isaković, 1964; Isaković and Janković, 1964; Cooper et al., 1966a), caecal tonsils (Cooper et al., 1966a; Panigrahi, 1970), medullary lymphoid follicles of the bursa (Janković and Isaković, 1964; Warner, 1967), and intestinal epithelium (Cooper et al., 1967; Panigrahi, 1970).

Lymphoid tissue associated with the paraocular and paranasal organs of the chicken has been identified by Bang and Bang (1968). These include lymphoid accumulations in the mucosa around lacrimal ducts, Harderian glands and villi

of their ducts, as well as beneath the epithelium of the lateral nasal gland ducts. Small lymphocytes contained within these structures may be thymus-dependent, but it has not yet been established.

Surgical removal of the chicken thymus within 24 hours of hatching results in a depletion of lymphocytes from the peripheral lymphoid tissue which is anatomically and functionally thymus-dependent (Cooper et al., 1966a). Thymectomy later in life or natural regression of the thymus does not grossly deplete thymus-dependent areas of small lymphocytes. Natural regression occurs at the onset of sexual maturity (Wolfe et al., 1962).

Structure and Development of the Bursa of Fabricius

The mature bursa contains lymphoid follicles consisting of an outer cortex of densely packed lymphocytes supported by a reticular network and separated from the diffuse lymphoid tissue and reticuloepithelial cells of the medulla by an epithelial zone and basement membrane (Payne, 1971). Epithelial and blast cells may pass from the medulla into the cortex (Ackerman and Knouff, 1959, as summarized in 1964).

The bursa originates as a small sac dorsal to the cloaca with proposed derivation from embryonic endoderm and/or ectoderm (Romanoff, 1960; Ruth et al., 1964). On the twelfth

day of embryonation, epithelial buds form in the undifferentiated lining of the inner surface of the bursa and vascularization commences (Ackerman and Knouff, 1963, as summarized in 1964). The epithelial buds give rise to lymphoid follicles and lymphocytopoiesis begins at 14 or 15 days embryonation (Ackerman and Knouff, 1959, as summarized in 1964; Papermaster and Good, 1962). The origin of bursal lymphocytes is not resolved, but there is evidence that yolk sac stem cells and possibly thymocytes "home" to and colonize the bursa (Moore and Owen, 1966; Linna et al., 1972; Potworowski, 1972; Toivanen et al., 1972a). The postembryonic stem cell originates from the bone marrow (Toivanen et al., 1972b). Presumably under the inductive influence of bursal epithelium, the stem cells differentiate into lymphoblasts which subsequently give rise to large, medium and small lymphocytes.

Removal of the bursa after one month of age and its normal regression around the onset of sexual maturity do not deplete bursa-dependent lymphoid tissue or alter the ability to produce antibody (Chang et al., 1957; Wolfe et al., 1957; Graetzer et al., 1963a; Cooper et al., 1969). The normal regression or involution of the bursa occurs at the onset of sexual maturity (Wolfe et al., 1962).

Relationship of the Bursa to Other Lymphoid Organs

The bursa is essential for the development of germinal centers and small lymphocytes (B cells) which proliferate and become plasma cells with the ability to produce immunoglobulins and antibodies (Chang et al., 1955; Van Alten et al., 1965; Takahashi, 1967; Seto, 1970b). It is not resolved whether the bursa only seeds lymphocytes peripherally (Woods and Linna, 1965) or also elaborates a hormone (Glick, 1960; Janković and Leskowitz, 1965; St. Pierre and Ackerman, 1965) which regulates the differention of non-bursa-derived lymphocytes into immunoglobulin producing cells.

The peripheral bursa-dependent system consists of small lymphocytes (B cells), plasma cells, and germinal centers (Warner, 1964; Cooper et al., 1966a). These are located in the red pulp of the spleen (Janković and Isaković, 1964; Cooper et al., 1966a; Potworowski, 1972), caecal tonsils (Cooper et al., 1966a; Warner et al., 1969; Panigrahi, 1970), and gastrointestinal tract associated lymphoid accumulations (Warner, 1965; Payne, 1971), as well as Peyer's patches (Panigrahi, 1970).

Lymphoid accumulations of germinal centers and/or plasma cells have been demonstrated also around the lacrimal ducts, Harderian glands and lateral nasal gland ducts (Bang

and Bang, 1968). Plasma cells within the paraocular and paranasal organs are thought to be responsible for immunoglobulin A (IgA) synthesis (Lebacq-Verheyden et al., 1972; Leslie and Martin, 1973). Relatively few large lymphocytes are found in the peripheral circulation of mature chickens (Lucas and Jamroz, 1961).

Although the chicken does not possess lymph nodes analogous to those found in mammals, there are small collections of lymphoid tissues (mural nodules) in the walls of lymphatic vessels. The mural nodules, which appear only after 27 days of age and develop germinal centers some 20 days later (Kondo, 1937, as cited by Payne in 1971; Biggs, 1957) contain supportive connective tissue and are vascularized by a plexus of small blood vessels. They lack a capsule and lymph sinuses, although germinal centers and plasma cells develop after antigenic stimulation (Good and Finstad, 1967, as reported by Payne in 1971).

Small foci of lymphoid tissue occur in other organs and tissues, including connective tissue, bone marrow, skin, liver, lung, kidney, pancreas, endocrine glands, peripheral nerves, larynx and trachea (as reviewed by Payne, 1971). The lymphoid elements may consist of small, medium, and large lymphocytes and occasional germinal centers. Whether they are normal lymphopoietic structures, a response to lymphomatosis virus (Oakberg and Lucas, 1949; Lucas and

Oakberg, 1950; Oakberg, 1950), or occlusion of blood vessels (Lucas, 1949; Thorbecke et al., 1957, as reviewed by Payne in 1971), or some combination of these remains unresolved.

Methods and Immunologic Effects of Thymectomy

Thymectomy in the chicken can be performed surgically shortly after hatching but because of the anatomical location of the thymus, complete thymectomy by blunt dissection is difficult, if not impossible (Warner and Szenberg, 1964a, b; Metcalf, 1964; Panigrahi, 1970; Panigrahi et al., 1971). Panigrahi et al. (1971), reported that thymic remnants do not undergo hypertrophy or hyperplasia in response to demand for increased function.

Functionally, thymectomized chickens have severely altered ability to reject homografts (Warner and Szenberg, 1962; Aspinall et al., 1963), have a reduced amount of experimental allergic encephalomyelitis (Janković and Išvaneski, 1963), and have reduced delayed sensitivity to diphtheria toxoid and tuberculin (Cooper et al., 1966a; Panigrahi, 1970; Fauser et al., 1973c). Compromised graft-versus-host reactivity by blood lymphocytes of thymectomized chickens has been reported (Cooper et al., 1966a).

In the chickens that have thymic damage as a result of hormonal bursectomy, homograft rejection is abolished

whereas graft-versus-host (GVH) reactivity is retained (Warner and Szenberg, 1962). Therefore, Warner and Szenberg (1962) postulated that GVH competence may not be entirely thymus-dependent. It may have originated from a third cell source or from thymus-dependent lymphocytes which migrate peripherally from the thymus early in embryonation. Sheridan and his associates in 1969 reported that thymectomy of donors in the GVH reaction increases mild splenomegaly caused by non-B-locus histoincompatability as compared with thymectomy plus irradiation which decreases gross splenomegaly resulting from B-locus histoincompatability.

The characteristic lesions of experimental allergic encephalomyelitis do not develop in thymectomized chickens (Blaw et al., 1967) and caseation necrosis of the spleen in experimental tuberculosis is decreased (Panigrahi et al., 1972).

In thymectomized chickens, the thymus-dependent lymphoid tissue is depleted of small lymphocytes. Depletions occur in the white pulp of the spleen, caecal tonsils, bursal follicles and intestinal epithelium. A reduction in the percentage of vascular lymphocytes in thymectomized chickens has been reported (Isaković and Janković, 1964; Janković and Isaković, 1964; Warner and Szenberg, 1964a; Jaffe, 1966). Cooper et al. (1966a), reported a decrease in the number of small lymphocytes in x-irradiated thymectomized chickens and no reduction in non-x-irradiated

thymectomized chickens. Fauser (1969) and Fauser et al. (1969 and 1973b) reported a decrease in total lymphocytes in x-irradiated and non-x-irradiated thymectomized chickens. Thymectomy of day old chicks resulted in a prolonged depletion of vascular lymphocytes (up to 5 months of age). Coupling x-irradiation with thymectomy did not result in further depletion of vascular lymphocytes.

The bursa-dependent functions remain in thymectomized chickens. Thymectomized chickens can produce antibody (Warner and Szenberg, 1962; Graetzer et al., 1963b; Isaković et al., 1963; Cooper et al., 1966a) and natural haemagglutinins (Graetzer et al., 1963b). Some exceptions have been reported. Graetzer et al. (1963b), reported a diminished antibody response by thymectomized chickens, and Cooper et al. (1966a) found thymectomized x-irradiated chickens produced quantitatively less antibody after experimental injection with antigen. The thymus-dependent system may be necessary for the recognition of certain substances as foreign which precedes the initiation of an immunologically specific response by either the thymus or bursa-dependent systems (Peterson et al., 1965). The cooperation of T cells in eliciting a B cell response has been demonstrated in chickens (McArthur et al., 1973; Weinbaum et al., 1973) and other animals. The thymus may also be necessary to regulate or terminate the degree of bursa-dependent antibody response. Wick et al. (1970b) reported

higher antibody titers in thymectomized chickens.

Recently, Rouse and Warner (1972a, b) reported functional thymectomy with antithymocyte sera, detected by loss of GVH reactivity.

Lymphocytes from a human infant with congenital absence of the thymus failed to react as T cells *in vitro* and *in vivo* (Lischner *et al.*, 1967).

Methods and Immunologic Effects of Bursectomy

Several methods for removal of the bursa are employed. Methods of bursectomy are evaluated as to effectiveness by observing depletion of bursa-dependent lymphoid tissue and absence of immunologic function. Reported methods of bursectomy include surgical bursectomy with or without x-irradiation thereafter, x-irradiation of the bursa itself, hormonal bursectomy, immunologically induced bursectomy in combination with another of the above methods, and cyclophosphamide treatment. The various methods of bursectomy vary in ease of performance as well as the degree of compromised bursa and bursa-dependent function.

The early studies of bursa function utilized surgical bursectomy (Chang *et al.*, 1955; Glick *et al.*, 1956). Surgical bursectomy within a few days after hatching resulted in reduction or absence of the primary antibody response (Mueller *et al.*, 1962; Janković and Isaković,

1966; Cooper et al., 1966a), and a partially active anamnestic production of IgM antibodies but no IgG antibodies (Claflin et al., 1966; Arnason and Janković, 1967). The development of natural haemagglutinins may be suppressed (Graetzer et al., 1963a, b; Mueller et al., 1964). Surgical bursectomy does not result in total agammaglobulinemia (Cooper et al., 1966a). Immunoglobulin levels in serum may be normal or show deficiency in IgG with increased IgM levels (Ortega and Der, 1964; Cooper et al., 1966a). This IgG deficiency may be apparent within the first few weeks after hatching (Meter et al., 1969), or may not become apparent until several months of age (Arnason and Janković, 1967). Since the discovery of chicken IgA, it has also been shown that IgA is deficient in serum of bursectomized chickens (Martin and Leslie, 1973). IgA production may be somewhat thymus-dependent in other animal species (Clough et al., 1971). Bursectomized chickens have a higher mortality due to diseases in which antibodies confer protection. These include diseases resulting from infections with Salmonella typhimurium (Chang et al., 1959), Leptospira icterohaemorrhagiae (Kemmes and Pethes, 1963, as summarized by Payne, 1971), Eimeria tenella (Challey, 1962) and Treponema anserinum (Soumrov et al., 1967 as summarized by Payne, 1971). Bursectomized chickens have a higher susceptibility to virus-induced myeloblastosis (Baluda, 1967).

There is a reduction of massive tumor formation of avian lymphoid leukosis by bursectomy as late as 4 months of age (Peterson et al., 1964; Peterson et al., 1966; Cooper et al., 1968). It is thought that the target cells of neoplastic transformation by the virus are the bursal lymphocytes (Peterson et al., 1964; Peterson et al., 1966; Cooper et al., 1968; Purchase et al., 1968).

Hereditary autoimmune thyroiditis characteristic of the obese strain of White Leghorns is decreased by bursectomy (Wick et al., 1970a). It is not resolved whether the thyroid damage is a result of antibodies being produced which are specific for thyroid cells and destroy them and/or whether the B cell infiltration of thyroid tissue which occurs, mechanically causes thyroid damage.

Bursa-dependent organs of bursectomized chickens have varying degrees of lymphocytic depletion (Isaković and Janković, 1964; Claflin et al., 1966; Cooper et al., 1966a; Arnason and Janković, 1967; Cooper et al., 1969).

The thymus-dependent functions reported to remain intact in bursectomized chickens are homograft rejection (Warner et al., 1962; Papermaster and Good, 1962; Aspinall et al., 1963; Isaković et al., 1963; Janković et al., 1963), delayed skin sensitivity reactions (Warner and Szenberg, 1962; Janković and Išvaneski, 1963; Janković et al., 1963; Cooper et al., 1966a), GVH reactions (Mueller et al., 1964;

Warner, 1965; Cooper et al., 1966a), and resistance to certain diseases requiring cellular immunity to confer protection (Pierce and Long, 1965; Longenecker et al., 1966; Long and Rose, 1970).

Because some bursa-dependent function persists following surgical bursectomy after hatching, x-irradiation to destroy bursal lymphocytes seeded peripherally prior to bursectomy has been used (Cooper et al., 1966a). Because the recommended dosage of irradiation is high enough to be lethal for 50 percent of irradiated chicks, alternative methods of achieving functional bursectomy have been developed.

Weber and Weidanz reported in 1969 successful functional bursectomy resulted from point irradiation of the bursa soon after hatching. Bursa-dependent areas of the spleen and caecal tonsils were depleted of germinal follicles and plasma cells. Surgical bursectomy in ovo at 17 days of incubation induces total agammaglobulinemia (Van Alten et al., 1965; Cooper et al., 1969) but it is more difficult than post-hatching bursectomy.

Hormonal bursectomy (Meyer et al., 1959; Mueller et al., 1960; Aspinall et al., 1961) is the prevention of embryonic bursal development by the use of certain hormones. The embryonating eggs may be dipped in a solution containing nortestosterone or embryos may be inoculated. Survival of chicks may be low if large doses of hormone or use early in

embryonation cause abnormal cloacal development resulting in fecal impaction after hatching. Hormonal bursectomized chicks may have some degree of thymic atrophy (Warner et al., 1962). Even if hormonal bursectomy does not result in agammaglobulinemia (Carey and Warner, 1964; Warner et al., 1969), the antibody response is severely reduced (Warner et al., 1969). Hormonally bursectomized chickens were unable to reject homografts of spleen cell suspensions (Papermaster et al., 1962). They also reported poorly developed thymus glands in hormonally bursectomized chicks. Sherman and Auerbach (1966) reported that doses of 19-nortestosterone sufficient to completely inhibit bursa development also retarded thymic morphogenesis.

Functional bursectomy can be induced by immunological destruction of immunoglobulin producing cells. Kincade et al. (1970) reported that injection of IgM anti-mμ-chain serum in ovo, and surgical bursectomy at hatching, resulted in long term reduction of serum IgM and IgG and absence of germinal centers in bursa-dependent peripheral lymphoid tissue. Injection of anti IgM serum at hatching with surgical bursectomy resulted in lower serum IgM. Surgical bursectomy at hatching, followed by several injections of anti-mμ-chain serum resulted in complete suppression of detectable serum IgM, IgG and IgA (Kincade et al., 1973).

Cyclophosphamide is an alkylating agent toxic to rapidly dividing cells and has been used as an anti-tumor

drug in certain human malignancies. Cyclophosphamide injected repeatedly prior to and immediately after hatching (Purchase, 1973) or used in combination with irradiation (McArthur et al., 1973) renders chickens totally agammaglobulinemic. Lymphoid development in the bursa is arrested and bursa weights and sizes are significantly smaller than normal (Seto et al., 1971; Toivanen et al., 1972a, b). Toivanen et al. (1972a, b) reported depletion of lymphocytes in thymus- and bursa-dependent areas in spleens of cyclophosphamide treated chickens.

In Vivo Tests of Cellular Immunity

In vivo, cellular immunity can be manifested and detected by several methods such as the tuberculin type delayed skin reaction, GVH reactivity, and homograft rejection.

Injection of an antigen intradermally into an animal sensitive to the antigen can cause a marked local reaction that is visible grossly as erythema and induration. The delayed type skin reaction must be differentiated from the antibody mediated Arthus reaction. High levels of circulating precipitating antibody are necessary for the development of an Arthus reaction. The formation of antigen-antibody-complement complexes is thought to initiate the local inflammatory reaction (Dvorak et al., 1970; Straus, 1972). In 1969 Eisen reported that antigen localized in

the polymorphonuclear leucocytes present at the reaction site. The Arthus type response starts about 2 hours after antigen injection, reaches a maximum by 5 hours and generally diminishes by 24 hours. A severe reaction may, however, persist for 24 hours or more, making it dificult to distinguish from a delayed skin reaction. Histologically the Arthus reaction is characterized by an acute inflammatory response with edema and leucocytic infiltration. Vasculitis, hemorrhage, and necrosis can occur if the reaction is severe (Jawetz et al., 1970).

The tuberculin reaction is the classic example and best studied of the delayed type skin reactions. The skin reaction is first visible after a few and up to 10 or 12 hours, reaching a maximum at 24 to 48 hours; it is a delayed reaction. An inflammatory response is critical to the expression of the skin reaction (Koster et al., 1971). Histologically, the inflammatory response and concommitant perivascular leucocytic infiltration of a delayed type skin reaction is biphasic. The first and minor phase at 3 to 4 hours is characterized by predominantly polymorphonuclear cells. At 5 to 6 hours polymorphonuclear leucocytes emigrate from the perivascular spaces. The second and major peak commencing at 8 hours is characterized by massive granulocytic cell and mononuclear cell emigration. By 24 hours, the polymorphonuclear leucocytes have emigrated and

the mononuclear cells remain immobilized around the blood vessels (Spector, 1967). The emigrating lymphocytes of the guinea pig appear to belong to a subpopulation with a characteristic for surface binding of Thorotrast particles (Wiener et al., 1971). Grossly the reaction may persist for some days. According to Eisen (1969) the antigen becomes localized on the cytoplasmic membrane of macrophages when cytophilic antibody is present.

The expression of the delayed skin reaction depends upon the presence of sensitive lymphocytes (Waksman et al., 1961; Hill, 1969) discussed below. Zakarian and Billingham (1972) reported that in leucopenic guinea pigs the ability to develop delayed skin reactions was impaired. The skin reaction occurs even in the absence of antibody (Holtzer and Winkler, 1967) and is immunologically specific. In the case of contact sensitivity to haptens, specificity of sensitivity was to the carrier protein and not the hapten (Gell and Benecerraf, 1961). Furthermore, both carrier specificity and strong delayed skin reactions resulted equally in guinea pigs sensitized with lightly or heavily substituted conjugates (Benacerraf and Levine, 1962).

Delayed skin sensitivity can be passively transferred from a sensitive to a nonsensitive individual only by living (Salvin and Nishio, 1972) sensitive lymphoid cells or some of their extracts or products such as transfer factor,

not by serum (Landsteiner and Chase, 1942; Chase, 1945; Lawrence and Pappenheimer, 1956; Najarian and Feldman, 1961; Lawrence et al., 1963; Salvin and Garvin, 1964; Lawrence and Valentine, 1970b). In the guinea pig, passive transfer of delayed sensitivity as measured by the delayed skin reaction was successful with lymphoid cells of the spleen, lymph nodes and peritoneal exudate but not with thymus or bone marrow cells of the same donors (Salvin et al., 1970). The duration of delayed sensitivity transferred in this manner varies among different animal species (Lawrence and Pappenheimer, 1956). In passively sensitized animals, a large proportion of mononuclear lymphoid cells at the reaction site are of host origin (Najarian and Feldman, 1961; Turk, 1962; Oort and Turk, 1963; McCluskey et al., 1963) and probably originate in the bone marrow (Lubaroff and Waksman, 1967, 1968). Kay and Rieke (1963) reported numerous donor lymphoid cells were present at the reaction site only if skin testing was done immediately following cell transfer.

The mechanism whereby a few sensitive lymphocytes can impart cellular sensitivity, or immunity, as demonstrated by a delayed skin reaction is not resolved. Sensitive lymphocytes may impart an informational molecule to host lymphocytes, similar to the mechanism, via RNA transfer, for passive transfer of antibody production (Abramoff and

Brien, 1968b). These substances may stimulate or instruct host lymphocytes to divide and give rise to a clone of sensitive host lymphocytes. The passive transfer of cellular immunity by leucocyte extracts and transfer factor (Lawrence, 1955; Lawrence and Pappenheimer, 1956; Jureziz et al., 1970; Paque and Dray, 1970; Rosenfeld et al., 1972) could support either hypothesis. Indeed, the mononuclear cells at the reaction site in passively sensitized guinea pigs have undergone recent proliferation (Spector, 1967). Alternatively, antigen may react with a few sensitive lymphocytes which in turn synthesize and release biological effector molecules which initiate a wide spectrum of nonspecific effects. These then may nonspecifically cause the subsequent cellular emigration, infiltration and mononuclear cell stasis at the site of the skin test. Injection of antigen intraperitoneally leads to a disappearance of macrophages in sensitive guinea pigs and may be analogous to the in vitro effect of migration inhibition factor (MIF) upon macrophages (Sonozaki and Cohen, 1971).

MIF, or a skin reactive factor which may be associated with MIF produced in vitro, can cause skin reactions when injected intradermally (Bennett and Bloom, 1968; Pick et al., 1971). A shorter time is required for the skin reaction. Histologically it is identified as being the delayed type. Another alternative for the mechanism of passive transfer of

sensitivity is that sensitive lymphocytes may induce synthesis of specific antigen receptor sites on nonsensitive T cells, converting them to sensitive T cells (Levin et al., 1973).

Intradermal testing of chickens is difficult because the site generally used is the lateral aspect of the wattle (Karlson, 1972). As an alternative site to the wattle, the dermis of the metatarsal foot pad can be used. The dermis of the wattle of a young male chicken is approximately 0.45 mm thick (Lucas and Stettenheim, 1972). The outside diameter of a 26 gauge ½ inch needle, recommended for avian tuberculin testing, is 0.45 mm (Becton-Dickinson, 1964, Appendix 1). There are no published reports of the dermal thickness of the foot pad (Lucas, 1973), but intradermal injection of the foot is easier than of the wattle.

Besides the Arthus type reaction, another type of skin reaction should be differentiated from the tuberculin type delayed skin reaction. The Jones-Mote reaction, or more recently termed cutaneous basophilic hypersensitivity (Bast et al., 1971), can be passively transferred with sensitive lymph node cells, not serum, requires protein or protein-antibody conjugates for induction of sensitivity, and skin reactions are delayed in onset without a persistent induration. Any induration is transient and can be elicited for only a few weeks after initial sensitization. The polymorphonuclear infiltrate at the site of the skin test consists

mainly of basophils. There is no detectable MIF produced by sensitive lymphocytes from guinea pigs with cutaneous basophilic hypersensitivity. Bast and his associates (1971) stress that a true delayed hypersensitivity can be induced experimentally only with complete Freund's adjuvant (CFA) or the equivalent. The wax D portion of the high lipid content of the Mycobacterium genus contained in CFA reportedly induces inflammation required for development of delayed sensitivity (Hiu and Amiel, 1971).

Immunologically competent cells responsible for transplantation rejection are operationally defined as those cells which are capable of initiating a GVH response. Immunologically mediated homograft rejection is primarily cell-mediated (Eddteston et al., 1969; Falk et al., 1970; Ferraresi et al., 1970; Starzl et al., 1970; Dormant et al., 1972; Hellström and Hellström, 1972). Histologically incompatible donor cells with this capability react immunologically to the recipient's tissues when inoculated into an immunologically incompetent recipient, and initiate a wide variety of pathological damage to the recipient. The severity of the reaction is determined by the size of the inoculum, the age of the donor, the cell type within the inoculum, the animal species test system, the age of the recipient and degree of incompatability between donor and recipient. The reaction of competent donor cells upon the recipient may cause death, splenomegaly, hepatomegaly,

anemia, pock formation on the chorioallantoic membrane of the embryonating chick, runting, or a combination of these (Simonsen, 1962). In contrast, if immunologically mature cells are inoculated into an immunologically competent non-isogenic recipient, the transplanted cells may in time be rejected. The rejection is by the recipient's immunologic mechanisms (Solomon, 1963).

The route of the administration of the cells which initiate GVH reactions is not critical. The cells appear to "home" to organs that normally contain leucocytes (Simonsen, 1962). An intravenous injection results in more generalized and rapid pathologic damage. It has generally been assumed that the destruction of host tissue is dependent upon proliferation of donor cells (Simonsen, 1962; Warner, 1964; Longenecker et al., 1970), and a proliferative response by lymphocytes appears necessary for the expression of GVH (Seto, 1968a). On the basis of characteristic GVH membrane lesions, Delanny and Ebert (1962) challenged this theory. Recent evidence shows that the recipient actively contributes to the ensuing pathologic lesions (Walker et al., 1972; Hartmann and Fisher, 1973). Killby and her associates (1972) proposed that, in the embryonating chick, the grafted immunocompetent lymphocytes provide the stimulus, presumably via RNA transfer, to cause the ensuing proliferative response by recipient hemopoietic stem cells. The proliferation of recipient, rather than or in addition

to donor cells, contributes to the pathologic damage of GVH disease (Walker et al., 1972). Induction of GVH reactions in 6-8 week old F_1 hybrid mice by injection of either of the parental strain's whole blood stimulated the recipients' spleen cells to become cytotoxic (Singh et al., 1972). They postulated that the cytotoxicity was both specifically directed by activated donor cells in the process of sensitization against host target cells and nonspecifically directed by host cells at damaging other host cells. Identification of the cell type which is immunologically competent in initiating GVH disease is of practical importance both in homotransplantation and in immunologic reconstitution of host deficiencies in immunocompetent cell populations. It has been established that where thoracic duct lymphocytes are used, a good correlation exists between the number of lymphocytes, particularly small lymphocytes, and the severity of GVH reactions in rodents. The cells collected from the thoracic duct are generally lymphocytes, 95% of which are the small long-lived recirculating type (Simonsen, 1962).

The chicken has not been shown to have highly functional lymph channels analogous to those of mammals and consequently whether long and short-lived lymphocytes exist is not known. Splenomegaly in embryonating chicks can be induced with as few as 30 blood lymphocytes (Terasaki, 1959)

but not by monocytes or red blood cells. The lymphocytes active in GVH reactions cannot be identified on the basis of morphology or bouyant density (Szenberg and Shortman, 1966).

The immunologic basis for GVH disease is the cell-mediated mechanism (Potworowski et al., 1971), but a humoral response can occur and contribute to rejection. Porter (as summarized by Simonsen, 1962) reported that grafted immunologically competent cells in rabbits produce humoral antibodies against the recipients blood group factors and postulated that this accounted for the resulting anemia often associated with GVH disease. Antibody production by donor cells, restimulated with antigen at the time of injection into a recipient, has been demonstrated in the chick embryo (Seto, 1970a). T cells are believed to be the type of lymphocyte that initiates the GVH reaction (Cooper et al., 1966a). The GVH reaction can be nullified or retarded by injecting the recipient with antibody to the donor tissues or by adding isologous (recipient type) adult spleen cells (Simonsen, 1962) to the inoculum to cause homograft destruction.

The genetic contribution to GVH reactivity is determined by transplantation or histocompatability antigens on clones of immunocompetent lymphocytes (Burnet, 1960, 1961, 1962 as reviewed by Simonsen, 1962; Simonsen, 1962). When transplanted to a non-isogenic recipient the genetically

determined antigenic differences of the recipient are recognized. This recognition must precede the ensuing proliferative response and subsequent destruction of the host or its organs. The GVH reaction is therefore a primary immunologic response requiring no known prior contact of competent cells with the foreign histocompatability antigens. Prior contact of the donor cells *in vivo* or *in vitro* with the recipient's transplantation antigens can accelerate the GVH reaction; however, the increase in severity is inversely related to the strength or dominance of the transplantation antigens, i.e., the degree of antigenic dissimilarity of donor and recipient cells (Nielsen, 1972).

The significance of the transplantation antigens in the GVH reaction was established in the mouse system and the major ones reside at the H-2 locus (reviewed by McDevitt, 1971). In chickens, the B blood group locus appears to be a major locus determining histocompatability (Longenecker *et al.*, 1973).

It is possible that thymic-dependent functions which include homograft rejection (Miller, 1962; Eddteston *et al.*, 1969; Falk *et al.*, 1970; Ferraresi *et al.*, 1970; Starzl *et al.*, 1970; Dormont *et al.*, 1972; Hellström and Hellström, 1972) and the GVH response are mediated by functionally different T cell subpopulations. There are subpopulations of T cells in mice with differing sensitivities and/or functionally restricted potential. The lymphocyte population

which mediates cytotoxicity as measured *in vitro* is not the same one responsible for GVH reactivity (Mage and McHugh, 1973). Cytotoxicity increased following immunization if there was a strong histoincompatability. This was shown by selective *in vitro* binding and cytotoxicity by nonadherent small lymphocytes to allogeneic target cells grown in monolayers. The nonbinding lymphocytes retained GVH reactivity but had no further cytotoxicity for additional target cells. It is not clear whether this indicates a difference between functionally differentiated (Sprent and Miller, 1970) and nondifferentiated T cells or in different functional capabilities of different T cell subpopulations (Bach and Brashler, 1970; Anderson *et al.*, 1972; Stites *et al.*, 1972).

The capability to initiate the GVH reaction as well as homograft rejection in the chicken is thymus-dependent (Cooper *et al.*, 1966a). The manner in which the thymus exerts its influence is not known. The blood lymphocytes increase in GVH immunocompetence with age and reach a plateau by 4 weeks of age (Seto, 1968b). Thymic lymphocytes generally initiate GVH reactions less effectively than lymphocytes from peripheral blood. Seto (1966) and Droege and his associates (1973) reported a higher proportion of thymus cells were GVH competent in older chickens than in young chicks. This GVH reactive population was characterized by high electrophoretic mobility recovered from low speed

centrifugation fractions of thymus cell suspensions (Droege et al., 1973).

The most sensitive means of assaying GVH reactions in the chicken is by measuring spenomegaly (Seto, 1966). Furthermore, intravenous injection resulted in greater splenomegaly than grafting techniques and the age of greatest sensitivity of the recipient was 13-14 days embryonation (Seto, 1966).

No reports were found in which cell populations from thymectomized or bursectomized chickens were tested for GVH reactivity as well as for immunologic function by one of the in vitro correlates of delayed sensitivity.

In Vitro Tests and Correlates of Cellular Immunity

Several in vitro correlates of delayed sensitivity have been reported. They will not be individually reviewed, but only enumerated to aid in reference. Incubation of sensitive lymphocytes with the sensitizing antigen can yield in addition to MIF other so-called biological effector molecules or lymphocyte factors which affect the behavior of macrophages, polymorphonuclear leucocytes, or in the case of interferon, other cells susceptible to infection by the same virus. These factors include a leucotactic factor for mononuclear cells (Ward and David, 1969; Ward et al., 1970). If antigen-antibody complexes are added to supernatants, a

chemotactic factor for eosinophils may be produced (Cohen and Ward, 1971; McGarry et al., 1971). Other factors produced in vitro are macrophage aggregation factor (MAF) (Lolekha et al., 1970), macrophage activating factor (Mooney and Waksman, 1970), cytotoxic factors (Granger and Williams, 1968; David and David, 1972), cloning inhibitory factor (Lawrence and Landy, 1969), skin reactive factor (Bennett and Bloom, 1968), mitogenic factor (Valentine and Lawrence, 1969), interferon (Green et al., 1969; Milstone and Waksman, 1970), antibody (Tsuchimoto et al., 1972; Meyers et al., 1972), and transfer factor (Lawrence and Valentine, 1970a). The characteristic spreading of macrophages in vitro on glass and plastic surfaces may also be inhibited (Fauve and Dekaris, 1968), but a name for a factor has not been given. The species in which some or all of the above have been demonstrated are humans, guinea pigs, mice, chickens, rats and rabbits. The biologic effector molecules listed above (with the exception of MAF, Nathan et al., 1971) may or may not all be produced simultaneously, or under identical culture conditions, and appear to differ functionally and physiochemically from one another.

An additional in vitro assay, the mixed lymphocyte reaction (MLR) differs from those listed above. It is not a test which ascertains if sensitization has occurred, even though during the MLR effector molecules such as mitogenic

factor (Gordon and MacLean, 1965) and MIF (Bartfeld and Atoynatan, 1970) may be produced.

The MLR tests for a primary reaction between genetically distinct cell populations (Maclaurin, 1972; Wagner et al., 1972). In the chicken, thymic medullary lymphocytes and splenic lymphocytes are equally active in the MLR (Weber, 1970).

When incubated in vitro with antigen for several days lymphocytes from animals with cellular immunity undergo a cell transformation or proliferation detected by observation of morphologic changes (Mills, 1966) or by tritiated thymidine uptake. The blastogenic or proliferative response requires the presence of macrophages (Hersh and Harris, 1968). A proliferative response may also occur by B lymphocytes stimulated in vitro with antigen. Whether the proliferative lymphocyte response is that of B or T cells is antigen dependent (Jacobs et al., 1972). A proliferative response not necessarily immunologically specific can be induced experimentally with substances such as phytohaemagglutinin (PHA) and keyhole limpet hemocyanin (KLH). PHA has mitogenic erythroagglutinating and immunogenic properties (Markley et al., 1972).

Tissue explants from sensitive chickens and/or guinea pigs (Aronson, 1931 and 1933; Rich and Lewis, 1932; Fabrizio, 1952) were early in vitro models of inhibition of cell

migration in the presence of the sensitizing antigen. Subsequently, oil-induced peritoneal exudate cells (PEC), consisting of macrophages and lymphocytes were incubated in capillary tubes with antigen added to the culture medium (George and Vaughan, 1962). Migration of PEC out from capillary tubes is inhibited if lymphocytes are from guinea pigs sensitive to the antigen, not from controls. Antigens unrelated to the sensitizing antigen or ones that induce only antibody production cause no release of MIF from lymphycytes as detected by inhibition of migration of PEC (David et al., 1964a, c; David and Schlossman, 1968). Soluble as well as particulate antigens have been used in vitro (Carpenter, 1963; Al-Askari et al., 1965; David and Paterson, 1965; Malloy et al., 1972).

In the presence of the appropriate antigen, lymphocytes from sensitive guinea pigs release a factor, MIF, which inhibits the migration of monocytes in vitro (Bloom and Bennett, 1966, 1968; Bloom and Jimenez, 1970). This factor inhibits the migration of macrophages from sensitive (direct MIF test) and nonsensitive (indirect MIF test) guinea pigs. The MIF produced by human lymphocytes inhibits guinea pig macrophage migration (Rajapakse and Glynn, 1970), an indirect test for MIF. In addition to inhibition of macrophage migration, lymphocyte migration in the presence of high amounts of serum may also be inhibited in migration in

guinea pigs (Halpern et al., 1967). But this has been challenged by Salvin et al. (1971). Only a few lymphocytes from a sensitive animal are necessary to detect the production of MIF (David et al., 1964b). The synthesis of MIF can be blocked in vitro by chemical blockers of protein synthesis (David, 1965). It does not exist preformed in the lymphocytes. Whereas antigen is reportedly necessary to stimulate in vitro MIF synthesis by sensitive lymphocytes, the action of MIF on macrophages from a nonsensitive individual (indirect assay) does not require additional antigen (Yoshida et al., 1972). Sensitive lymphocytes in the MIF test require a given amount of antigen to continue elaborating MIF. Escape from migration inhibition by macrophages can occur after prolonged in vitro culture but inhibition can be restored by addition of more antigen (David et al., 1964b; Nathan et al., 1971).

The action of MIF on macrophages may require cytophilic binding of MIF to macrophages. Bartfeld and Atoynatan (1971) were able to block migration inhibition of MIF by treating macrophages with N-acetylcysteine which inactivates the binding site for MIF on the heavy chain of guinea pig macrophage-associated cytophilic antibody. In guinea pigs, delayed skin reactions can be elicited sooner after sentitization than can in vitro MIF production be detected (Ferraresi et al., 1969).

There is no method yet to distinguish single lymphocytes of a given sensitivity (Gowens and McGregor, 1965). However, sensitive T cells support RNA virus replication (Bloom et al., 1970; Bloom, 1971). Currently, the use of the term sensitive lymphocytes indicates lymphocytes from an animal with delayed sensitivity, without inference that all the lymphocytes present are sensitive to a given antigen (David and David, 1972). T lymphocytes, in general, can be identified in humans, rabbits, guinea pigs, rats and mice by their characteristic light refringence (Pompidou and Schramn, 1971) and in certain species by the presence of a characteristic T antigen (Potworowski and Nairn, 1967; Owen and Raff, 1970; Malchow et al., 1972; Jacobs et al., 1972). T cells have not been shown to bind antigen by means of cell-associated antibody. This property is used to identify B cells (Wigzell and Andersson, 1969). However, immunoabsorption at 37 C on mouse fibroblasts by rat lymphocytes via their receptor to strain specific antigens on the mouse fibroblasts has been reported to be a property of T cells in rats (Wekerle et al., 1972). These specifically immune T cells have a short life span and a rapid turnover in mice (McGregor et al., 1971).

Sensitive lymphocytes from PEC, lymph nodes, blood, and spleen of many mammals elaborate MIF (Salvin et al., 1970; Winkelstein, 1972), whereas thymus and bone marrow cells do

not (Winkelstein, 1972). Spleen cells from sensitive chickens elaborate MIF (Morita and Soekawa, 1971; Zwilling et al., 1972). Peripheral blood has been used as the source of sensitive lymphocytes from man (Clausen and Søborg, 1969; Kaltrieder et al., 1969; Mookerjee et al., 1969; Bendixen and Søborg, 1970; Tarnvik, 1970; Clausen, 1971) and the chicken (Fauser et al., 1973a; Fauser et al., 1973c). In vitro culture of blood leucocytes is more difficult than culture of leucocytes from other sources. Serum used in culture is reported to be one critical factor. Although serum free medium is available for in vitro culture of chicken leucocytes (Weber, 1970), it cannot be used successfully if sensitive cells are concommittantly stimulated with antigen (Kirchner and Oppenheim, 1972).

Peritoneal exudates, or chicken abdominal exudates, are difficult to obtain and peripheral blood is not. Another advantage of using blood experimentally as compared to spleen cells (Morita and Soekawa, 1971, 1972) is that the same animal can be used repeatedly, as his own control, before and after the induction of delayed sensitivity.

Techniques other than explants and capillary tube migrations have been reported (Salvin and Nishio, 1969; Smyth and Weiss, 1970; Mallmann et al., 1971; Houck and Chang, 1973; Harrington and Stastny, 1973). These methods utilize, in one form or another, a monolayer or droplet of lymphocytes and macrophages or both to which various test

antigens and culture medium are added. Serum, but not complement, is required in most of these assays (George and Vaughan, 1962; Bennett and Bloom, 1968; David and David, 1972).

Lymphocyte function(s) in delayed sensitivity is(are) not completely understood. The sensitivities and conditions necessary for elicitation of a response in vitro as compared to in vivo vary (Thomas et al., 1971). If the various in vitro correlates of cellular immunity are duplicated in vivo, several in vivo phenomena may possibly be explained. These phenomena include the following: (1) the reticuloendothelial systems activation and mitosis of macrophages (North, 1970) following infections which lead to delayed type sensitivity (Mackaness, 1971; Youmans, 1971), (2) the presence of recipient cells not donor cells, at the site of a skin test in passively transferred delayed sensitivity, and (3) the accumulation of mononuclear cells which give rise to granulomas and/or tissue destruction (Ruddle and Waksman, 1967) in certain diseases caused by intracellular parasitism.

Very few specifically sensitive lymphocytes are required to affect and involve, by a mechanism or means not yet fully understood, many nonsensitive cells to participate in and cause many nonspecific reactions which are nonetheless immunologically specific both in induction and memory (McGregor et al., 1971). This amplification of immunologically activated responses, known collectively as cellular

immunity, and independent retention of immunologic memory is an obvious advantage to biological specialization and conservation.

Cell-mediated Immunity and Its Clinical Manifestations

Cell-mediated immunity which is mediated by and resides in the sensitive lymphocyte is involved directly and indirectly in many disease processes. It is manifested by both protective and untoward reactions. The clinical manifestations of cell-mediated immunity are so varied that only recently, with the advent of *in vitro* tests, have the breadth and complexity of T cell-mediated reactions become more apparent.

It has been recognized since the time of Koch (Mackaness 1964, 1969, 1971), that delayed hypersensitivity, exemplified by the tuberculin type skin reaction, results from intracellular infections with many fungal, bacterial and protozoan agents. The various *in vitro* correlates of cellular immunity have begun to enumerate the physiologic repertoire of reactions associated with cellular immunity. Functional and/or structural defects in the T cell in immunologic deficiency diseases may be associated clinically and be responsible for increased susceptibility to recurrent or chronic infection with certain group of microorganisms (Chilgren *et al.*, 1969; Mendes and Raphael,

1971). Many so-called autoimmune diseases, both naturally occurring and experimentally induced, are cell-mediated (Janković and Mitrovic, 1963; David and Paterson, 1965; Brostoff et al., 1969; Behan et al., 1970; Werdelin and McCluskey, 1971).

It has been proposed that in man heavy irradiation of the thymus gland during childhood predisposes to the development of neoplasms (Janower and Miettinen, 1971). Cellular immunity to tumor specific antigens has been shown in naturally occurring and experimentally induced tumors (Kronman et al., 1969; Sojogren and Borum, 1971; Wolberg, 1971; Churchill et al., 1972) and regression of certain human neoplasms by a cell-mediated process has been reported (Stjernsward and Levin, 1971; DiSaia et al., 1972). Intradermal injection in guinea pigs of MIF with hepatoma tumor cells suppressed local tumor formation (Bernstein et al., 1971). Mice infected with leukemia virus and sensitized with CFA had no detectable MIF in vitro (Friedman and Ceglowski, 1971). Similarly, Hodgkin's disease patients have decreased MIF production in vitro (Churchill et al., 1971).

Antibody may block the cytotoxic effect of immune T lymphocytes by a process referred to as immunologic enhancement, and may mask a functionally capable cell-mediated immunity (Hellström and Hellström, 1972). The antibody

coating the target (tumor) cells prevents the cell-mediated destruction by T lymphocytes (Prehn, 1971).

The immune system may be functionally depressed secondarily by infections (Notkins et al., 1970; Purchase et al., 1968) or the cells of the immune system may be infected.

Indications are that the lymphoid leukosis virus of chickens replicates within the cells of the bursa prior to its spread systemically. Surgical bursectomy prevents the cycle of the infection (Peterson et al., 1964).

The etiologic agent of Marek's disease of chickens is a group B virus which is a cell-associated herpes virus. The disease is lymphoproliferative (Calnek and Witter, 1972). Whether the disease is primarily neoplastic or inflammatory in nature has been widely debated (Purchase, 1972). Marek's disease (MD) infected chickens have increased quantities of circulating globulin (Purchase, 1972) and maternal antibodies appear to confer protection against naturally occurring spread to young chicks (Calnek and Witter, 1972). General immunologic function is depressed as determined by susceptibility to coccidia, and decreased antibody titers (Burg et al., 1971) with concommitant elevated gammaglobulin levels (Calnek and Witter, 1972). Neither thymectomy or bursectomy prevents the disease. Chickens with MD are reported to have fewer thymus-dependent lymphoid areas in the spleen (Evans et al., 1971).

Gonadal tumors in MD infected chickens consisted of T cells and there is a B cell lymphocytosis in the blood (Hudson and Payne, 1972). The presence of T lymphocytes in MD tumors has been confirmed and extended to include tumors of the spleen, ovary, and nerves as well as blood (Rouse et al., 1973).

MD viral antigens induce delayed hypersensitivity skin reactions and MIF production in vitro (Dawe et al., 1971; Fauser et al., 1973a; Fauser et al., 1973c). It is not resolved if the pathogenesis of MD is partially autoimmunity (Rouse et al., 1973), if cellular immunity has a role in the natural age regression of MD lesions (Calnek and Witter, 1972), or if the T cell response has any significant role in MD.

The induction of cell-mediated immunity is one possible means for prevention or treatment of diseases in which antibodies are not protective. It can be by vaccination and/or passive transfer of immunity by sensitive lymphocytes, their extracts, or products (Lawrence, 1955; Bernstein et al., 1971; DiSaia et al., 1972). Vaccination with Bacille Calmette-Guerin (BCG), for tuberculosis of man, a disease in which antibodies do not confer protection, is done routinely in many countries. The passive transfer of cell-mediated immunity is still experimental (Levin et al., 1973).

Whether the partial protection given by BCG is specific is not resolved. One possibility is that the sensitive

lymphocytes, established by BCG vaccination, when stimulated *in vivo* with antigen, recruit effector cells such as macrophages to the foci of infection (Mackaness, 1971). The macrophages in turn can sequester and kill intracellular parasites. Another suggestion has been that the inflammatory reaction, which is initiated and maintained by BCG infection, may augment resistance by nonspecifically stimulating macrophages to a hyper-reactive state (Dumonde, 1967; Melnick, 1971). This in turn may enable macrophages to destroy tubercle baccilli, nonrelated organisms, and, perhaps even neoplastic cells.

It is not known how vaccination of chickens with herpesvirus of turkeys (HVT) protects against Marek's disease. Protection may result by a nonimmunologically mediated mechanism(s) or by immunologically mediated humoral and/or cellular immunity. The host parasite relationship between the virus and infected cells of MD can serve as a model for the study of other infections of a chronic nature as well as neoplastic diseases of mankind. It is worthwhile to determine whether cell-mediated immunity results from infection with MDV.

The chicken model is suitable for studying cell-mediated immunity and humoral immunity singly. Of specific interest is the perfection of a technique, suitable for use with chickens, to study cell-mediated immunity *in vitro*.

Whether MIF production is thymus-dependent can be tested. There are reports of MIF production by chicken leucocytes (Morita and Soekawa, 1971; Zwilling et al., 1972). Sufficient numbers of leucocytes for in vitro assays have been obtained from spleens. In the absence of abundant numbers of lymph nodes, blood is a more feasible source of leucocytes for repeated MIF assays. There are a number of advantages to adapting the MIF procedure for use with blood leucocytes. Each chicken can be used as its own control, reducing the size of experimental error due to individual chicken differences. Leucocytes can be tested for MIF production prior and subsequent to sensitization. Additional assays for T cell function, namely skin reactions and GVH reactions, can be used as in vivo tests for sensitive T cells in the chicken.

Because BCG as a vaccine stimulates cell-mediated immunity to tuberculosis of man and it is known that it induces as least delayed sensitivity in chickens, it can be used as a positive control for cell-mediated sensitivity in the chicken as well. BCG is not pathogenic for chickens, but can cause a severe inflammation and delayed sensitivity. The MDV is less pathogenic for chickens after they are 8 weeks of age (Witter et al., 1973). MDV infection can be induced in the adult chicken without high mortality.

The discovery that the bursa of Fabricius in the chicken is the central lymphoid organ for the immunoglobulin

producing system has stimulated the subsequent search for the mechanisms underlying the establishment of and *modus operandi* of the thymus-dependent cell-mediated immune system.

MATERIALS AND METHODS

Chickens and Chick Embryos

The White Leghorn chickens used in all experiments were either F_1 hybrids of Regional Poultry Research Laboratory Line 15x7, offspring of line 6x7 ♂♂ crossed with line 6 ♀♀, or embryos of line 15, subline 1 and 4 (Stone, 1974).

Collection of Blood for Tissue Culture and Test Serums

Serum used in tissue culture was separated from blood of specific pathogen free (SPF) chickens of varying genetic background. Chickens had been maintained separately for genetic studies and blood was obtained after their SPF status was confirmed (Stone, 1974).

Chickens were exsanguinated by cardiac puncture. Blood was conveyed by needle, plastic tubing, and a vacuum pump from the heart to glass bottles which were laid on the side until the blood clotted. Bottles were shaken to dislodge the clot, and stored at room temperature overnight. The following day, fluid was decanted, pooled, and centrifuged at 1,000xg for 15 minutes. The serum was decanted from the sterile centrifuge tubes and filtered through a

0.45 μ Millipore filter (Millipore Filter Corp., Bedford, Mass.). The serum was tested for sterility by incubating 0.5 ml serum in 4.5 ml each tissue culture medium (a 3:5 ratio of F10, M-199) (Appendices 2 and 3) and tryptose phosphate broth for one week. Only sterile sera were used in tissue culture.

Serum was dispensed in either 20, 30 or 45 ml samples in sterile plastic screw cap tubes and stored at -20 C until used in tissue culture medium. Throughout each experiment serum from the same pooled lot was used.

Blood, used as a source of leucocytes and test serum, was taken from the brachial wing vein with syringe and 22 gauge 1 inch needle. Test serum samples were separated from 2 1/2 to 5 cc of blood.

Viable leucocytes were separated from 10 ml brachial wing blood collected into 0.05 cc heparin (sodium heparin, USP 1,000 units/ml, Fellows Medical Manufacturing Incorporated, Oak Park, Mich.) with a 10 cc syringe and 22 gauge 1 inch needle.

Experimental Sensitization

Chickens were sensitized by injection with viable BCG in CFA, CFA, and/or HVT. Those sensitized with BCG were injected subcutaneously with a total of 1 ml containing 5 mg wet weight viable BCG in an equal amount with incomplete

Freund's adjuvant (IFA) (#063760, Difco Laboratories, Detroit, Mich.).

CFA (Control 516160 Difco Laboratories), a total of 1 ml in an equal amount with sterile saline, was injected subcutaneously. Cloned GA strain of MDV (Purchase et al., 1971) was injected subcutaneously at a dose of $7x10^3$ plaque forming units (PFU). HVT, $1.4x10^5$ PFU, was injected subcutaneously.

In Vivo Techniques

A. Thymectomy

Thymectomy was performed within 24 hours after hatching. The chicken was anesthetized with 0.05-0.08 cc Combuthal (Diamond Laboratories Inc., Des Moines, Iowa) administered intra-abdominally. An incision approximately 4 cm long was made on the dorsal surface of the neck and each lobe of the thymus, with surrounding connective tissue and fat deposits removed by blunt dissection. The incisions were closed with Michel wound clips (Propper Manufacturing Co., Inc., Long Island City, New York) which were removed 3 weeks later. Aseptic techniques were used. Antibiotics were not administered postoperatively.

B. Bursectomy

Bursectomy was performed within 24 hours after hatching under anesthesia described above. An incision

approximately 2 cm long was made immediately dorsal to the cloaca and ventral to the tail. The bursa of Fabricius was dissected from the surrounding connective tissue and removed at the stalk. The incision was not closed and antibiotics were not administered postoperatively. Aseptic techniques were used.

C. Graft-versus-host Assay

Leucocytes were purified by the method described below in Leucocytes for Migration Inhibition Test except that they were kept in sterile plastic tubes at 4 C, not attached to petri dishes. On the basis of the total mononuclear leucocyte counts using Trypan Blue dye (Grand Island Biological Company, Grand Island, New York) in phosphate buffered saline (PBS) diluent, the final volume was adjusted to contain 10^6 mononuclear leucocytes/0.05 cc. Embryos of fourteen day embryonating eggs (line 15 subline 1 and subline 4) were used as recipients. The chorioallantoic vein was located with the aid of a candler and its location marked on the shell. The shell, but not the shell membrane, was cut with a small electric disc saw and removed from the shell membrane with a sterile 26 gauge 1/2 inch needle. The eggs were stored, cut surface facing upward, for at least 20 minutes to allow the embryos to become relatively quiescent. Thereafter, the exposed shell membrane was covered with sterile mineral oil to increase transparency.

One million viable mononuclear leucocytes were injected in a 0.05 cc volume of complete tissue culture medium into the chorioallantoic vein. Thirty gauge 1/2 inch needles were used to inject donor leucocytes, with the direction of the blood flow. A momentary clearing of the contents of blood from the vein aided in visually ascertaining the success of intravenous injection. Leucocytes from each donor were injected into a minimum of 10 embryos. The embryonating eggs were incubated in a single stage Jamesway incubator at standard operating conditions regarding temperature, humidity, air flow and rotation (Jamesway Incubators, Appendix 4).

Nineteen day embryonating eggs to be assayed for GVH splenomegaly were removed from the incubator and stored overnight at 4 C. The intact spleens were removed from embryos. Each spleen was blotted with dry filter paper and stored individually in plastic preweighed weighing boats. Five weighing boats were placed in covered plastic petri dishes containing moisture-saturated filter paper. The weighing boats containing spleens were weighed individually within minutes of harvesting on a Mettler balance. Weights were recorded to four decimal places.

D. Skin Testing

Skin tests were by intradermal (ID) injection in one wattle of 0.05 ml solution of antigen in sterile PBS.

In one experiment a skin test with sterile PBS was performed one week prior to skin testing with Band-24 (B-24). The other wattle served as a control. The degree of swelling was observed at 20 minutes, and periodically thereafter at 2, 5, 24, 48, 72 and 96 hours post injection. The skin reactions were recorded on a relative scale from 0 to 4+. A reading of 1+ indicated that the induration and/or inflammation of the wattle was twice as thick and a 4+ indicated that it was five times as thick as the uninoculated control wattle.

In Vitro Techniques

A. Leucocytes for Migration Inhibition Test

The procedure for the MIF test was an adaption of that described by Mallmann et al. (1971). Ten ml of heparinized blood was poured through glass wool (Corning Pyrex Brand 3950 Fibre Glass No. 7220, Corning, New York), centrifuged at 1,000xg for 20 minutes and the leucocyte rich buffy coat was drawn into sterile heparinized capillary tubes. The tubes were sealed with wax, centrifuged and broken at the cell-plasma interphase. The leucocytes were removed with a syringe and 26 gauge needle and diluted to 10^8 cells/ml in tissue culture medium. Drops approximately 4 mm in diameter were placed on 35 mm diameter plastic petri dishes (Falcon Plastics, Los Angeles, Calif.). After 10 minutes the non-attached cells were rinsed off and 2 ml of antibiotic-free

F10-M199 tissue culture medium (Witter et al., 1969) containing 15% serum from SPF chickens instead of calf serum, and antigen as indicated, was added. Duplicate plates usually containing 8 spots per plate were prepared for each antigen and control culture. The diameters of the spots of attached leucocytes were measured with an ocular micrometer before and after 24 hours of incubation in a CO_2 incubator, 5% CO_2 in air at 37 C. The distance migrated was calculated by subtracting the diameter of the spot prior to incubation from the diameter of each spot after incubation. Viability of cells inhibited in migration was shown by Trypan Blue exclusion.

B. Antigens

Old Tuberculin (OT, serial number 9537, USDA) was used undiluted, for skin testing at 145 µg protein in a volume of 0.05 ml and in vitro at either 25 µg/ml or 50 µg/ml culture medium. B-24, a protein isolated from culture filtrates of BCG (Roszman et al., 1968; Fauser et al., 1969) (supplied by V. H. Mallmann, Assoc. Prof., Department Microbiology and Public Health, M. S. U., East Lansing, Mich.), was used. The skin testing dose was diluted in sterile PBS to contain 3.5 µg protein in 0.05 ml PBS.

In vitro 2 µg protein/ml culture medium was used.

Crude MDV antigen was prepared as previously described (Chubb and Churchill, 1968). The GA strain of MDV was

propagated in chick embryo fibroblasts in a mixture of F10-M199 tissue culture medium supplemented with SPF serum. Crude MDV antigen was used for skin testing undiluted and *in vitro* diluted 1/24 in tissue culture medium.

Antigen A (Churchill *et al.*, 1969; Purchase, 1970) was obtained from Dr. P. A. Long prepared as previously described (Long, 1973). The DEAE sephadex A-25 eluate was dialyzed against PBS and filtered through 0.45 μ Millipore filter pretreated with chicken serum (Ver *et al.*, 1968). Antigen A was used undiluted, 0.05 ml/chicken, for skin testing. The antigen was used *in vitro* at a 1/8 dilution, the dilution at which a strong precipitin line was found with standard MDV antiserum in double diffusion in agar coated slides.

C. *Antibodies*

Precipitating antibodies were detected by double diffusion in agar coated slides with a two-fold concentration of second strength purified protein derivative containing 0.01 mg protein (PPD, Parke-Davis, Detroit, Mich., #NDC 71-1298-1 Bio. 484) as antigen. A 1% agar was prepared in an 8% NA Cl solution and poured onto clean glass slides. Holes were punched in the solidified agar, filled with test sera or plasma, according to the method of Cho and Kramer (1970), and incubated in a humidified chamber for at least 48 hours to allow precipitin lines to develop.

The indirect fluorescent antibody method was performed according to the procedure described by Purchase (1970) and Solomon et al. (1971). Chick kidney cells were grown on coverslips until a monolayer had formed (Calnek and Madin, 1969). Monolayers were infected with either HVT or MDV (GA strain). During primary foci formation, the coverslips were removed from the culture medium, washed in saline, and fixed in acetone. The air dried coverslips were stored at -20 C until used. The coverslips were divided by latex paint into 4 equal areas. Test serums, all SPF serums, and standard positive and negative control serums were randomly assigned by code, 4 per each coverslip. After incubation of coverslips with a 1/20 dilution of coded test sera for one-half hour, in a humidified chamber, the coverslips were washed for 15 minutes in FTA Hemagglutination buffer (Bioquest, Cockeysville, Maryland). Thereafter, the buffer was removed, and coverslips were covered with fluorescein labeled horse anti-chicken serum and incubated one-half hour. They were then washed for 15 minutes with FTA buffer. The coverslips were rinsed in distilled water, mounted on glass slides with Elvanol (Polyvinyl Alcohol Grade 51-05, DuPont, de Nemours and Co., Wilmington, Delaware; Rodriguez and Deinhardt, 1960), monolayer surface down, and viewed in a fluorescence microscope. The degree of fluorescence was scored by a relative scale from 0 to 4+.

D. Bacteriological Techniques

Bacteriological techniques were used on liver and spleens from one lot of chickens to isolate acid fast bacilli. Fragments of the liver and spleen were ground, in a mortor with a pestle, in nutrient broth. An equal amount of 1N NaOH was added to the tissue homogenate and after 15 minutes at room temperature, was neutralized with 1N HCl. The samples were centrifuged and the sediment was seeded on 5 tubes each of Dubos oleic agar and Lowenstein-Jenson media (Difco Lab., Detroit, Mich.). The tubes were examined for growth on the first of each month thereafter. Before being discarded, acid fast stains were made on smears taken from the surface of the tubes to confirm no growth.

Postmortem Examinations

At the termination of each experiment, chickens were carefully examined for thymic remnants when appropriate and scored as normal thymus or as either complete or incomplete for thymectomy. All the viscera and brachial, sciatic and celiac nerves were examined for gross lesions indicative of MD. Viscera were examined for tuberculosis-like lesions when appropriate.

Liver, spleen and serosa samples from all BCG and CFA inoculated chickens were collected, prepared for histopathologic examination, and stained with new fuchsin-hematoxylin-eosin (Willigan et al., 1961) according to methods

used by Panigrahi (1970). Tissues were examined for acid fast bacilli, for the presence of giant cells, and granulomatous lesions.

Statistical Analyses

Data were analyzed by the basic analysis of variance technique aided by the USDA-ARS data Systems Application Division. To accommodate missing data or unequal sample sizes, the least-square analysis (Kirk, 1968) was employed. Where possible, Bartlett's test for homogeneity of variance (program BMDP9D) was used.

The analysis of leucocyte migration of the thymectomized or bursectomized chickens utilized a split plot model in a random design with repeated measurements of animals (Gill, 1971). Alpha levels, the probability of incorrectly inferring the existence of a true effect of treatment were established for each experiment to reflect the biologically justifiable expectations within each experiment and, unless otherwise specified, were alpha 0.05.

Experimental Designs

A. Experiment I

The objectives of this experiment were (1) to determine if migration of leucocytes from control, CFA (containing *Mycobacterium butyricum*) sensitized and BCG (*M. bovis*) sensitized chickens are inhibited by two concentrations of

OT and by B-24 (prepared from BCG), and, (2) to determine the concentration of antigen needed to inhibit leucocyte migration but retain cell viability in vitro.

Day-old male chicks, line 15 x line 7 offspring, were identified by wing bands assigned randomly and housed in Horsfall/Bauer stainless steel isolators (Hartford Metal Products, Inc., Aberdeen, Md.) at the United States Department of Agriculture Regional Poultry Research Laboratory (USDA-RPL) at East Lansing, Michigan.

Leucocytes from the chickens, when two months old, and 2 months after sensitization, were tested for MIF production. Leucocytes from each chicken were cultured in duplicate plates for each, no antigen, 25 µg OT/ml culture medium, and 50 µg OT/ml culture medium.

Twenty adult chickens were randomly assigned to one of three experimental groups. Serum samples were collected. One group of 8 chickens received viable BCG in IFA, another group of 8 chickens were injected with CFA and 4 chickens served as controls. No more than 4 adult chickens, each with the same inoculum, were housed in each isolator.

Two months after sensitization, leucocytes were obtained two times from chickens in all lots and tested for migration inhibition with two concentrations of OT used in tissue culture. Two additional test plates with B-24 at 2 µg protein/ml culture medium were prepared from each chicken's leucocytes during the second test.

Three months after sensitization, all chickens were skin tested with B-24. One wattle was injected with 0.05 ml sterile PBS, as the control. Two weeks later skin testing was repeated with OT.

At the termination of the experiment all chickens were bled for serum, and killed by intracardiac injection with air.

The presence or absence of lesions involving internal organs was recorded. Samples of lesions, liver, and spleen were taken for histopathologic examination and bacteriologic reisolation.

All serums were tested for precipitating antibody with PPD.

B. Experiment II

The objective of this experiment was to determine whether MD can induce delayed sensitivity to a soluble antigen of MDV.

Six-month old chickens housed as in Experiment I, were bled for serum and their leucocytes tested for inhibition of migration with crude MDV antigen and OT. Each chicken was randomly assigned to one of three treatment groups, 6 chickens each. The three lots consisted of (1) Controls, (2) MDV in CFA injected, and (3) MDV injected chickens housed with noninoculated chickens to simulate conditions for the natural spread of MDV by contact exposure.

One to three months later, leucocytes from surviving chickens were tested for migration inhibition with crude and partially purified A-antigen preparations and OT. They were skin tested with both MDV antigens, and later with OT. At 11 months of age, chickens were bled for serum and the sera were tested for the presence of MDV antibody by the indirect fluorescent antibody test.

At the termination of the experiment the brachial, sciatic, and celiac nerves were collected and prepared for histopathologic examination if no gross MD lesions were evident.

C. Experiment III

The objectives of this experiment were to determine whether the potential to initiate GVH disease was eliminated from leucocytes derived from thymectomized donors and to determine whether *in vitro* production of MIF was thymic-dependent.

The study included intact, bursectomized and thymectomized White Leghorn progeny of the USDA-RPL line 6 x 7 ♂♂ x line 6 ♀♀. Surgical bursectomies and thymectomies were performed within 24 hours after hatching. The chickens were housed initially in a battery brooder, then in cages until they were transferred to Horsfall/Bauer isolators (USDA-RPL) at 6 months of age.

The GVH assay was performed as described above. Recipient embryos were either line 15 subline 1 or subline 4. Eight-month-old donor chickens belonged to the following treatment groups: intact, bursectomized, or thymectomized.

The GVH assay was repeated 6 weeks later using six thymectomized and six intact donors. Five recipient embryos each of line 15 subline 1 and line 15 subline 4 were injected with each of the donor's leucocytes. Each time cells were prepared for a GVH assay, the plasma was tested for MDV antibody by the fluorescent antibody test. In addition, leucocytes from dams of the same subline as the embryo recipients were tested for GVH reactivity as a control.

Prior to sensitization at 9 months of age, blood leucocytes were tested twice at 3 week intervals for inhibition of migration. The serum was tested for precipitating antibody to PPD and MDV and all chickens were skin tested with 3.5 μg protein of B-24. The same chickens were subsequently sensitized intra-abdominally with 5 mg wet weight BCG in IFA. Three weeks after sensitization their leucocytes were again, twice, at 3 week intervals, tested for migration inhibition. Antibody determinations were made as above, and chickens were skin tested with B-24 and with OT.

One week after skin testing with B-24, all chickens were vaccinated with HVT, and tested for HVT antibody 6 weeks later at the time of skin testing with OT.

Chickens were asphyxiated by CO_2. At necropsy, completeness of thymectomy was determined, and sections of liver, spleen, and any grossly visible granulomatous lesions were processed for histopathologic examination.

RESULTS

A. Experiment I

The results of *in vitro* migration of leucocytes from control (nonsensitized) chickens in the absence of antigen and with the addition of 25 and 50 µg OT were analyzed using the complete block design for a random model (Steel and Torrie, 1960). The data was first transformed by adding the value five to each migration measure and the analysis was performed using the data from six chickens with no missing data. Table 1 enumerates the transformed measurements of the distance migrated in micrometer units after 24 hours of *in vitro* culture by leucocytes with and without antigen added to the culture medium. The analysis of variance obtained from the data is presented in Table 2. The analysis indicated no detectable effect by antigen on leucocyte migration. The distance of migration varied among individual chickens. The lack of detectable interaction between chickens and antigens indicated that the variability for the individual chickens was not antigen dependent. There were no significant differences between duplicate plates. The greatest variability was among spots within duplicate plates. The variation of total migration for all spots in all plates

Table 1. Migration in vitro of leucocytes from control chickens with and without antigen in the culture medium.

Chicken		No antigen	25 µg OT	50 µg OT
834	plate I :[1]	25 25 25 25 15 15 10 15	25 25 15 20 15 15 20 20	10 15 15 15 15 15 15 10
	plate II:	15 15 20 20 15 15 25 15	25 15 25 25 25 20 15 20	15 15 15 15 15 25 15 15
843	plate I :	25 25 20 20 30 20 20 20	25 20 20 15 25 25 15 25	05 05 10 15 10 15 20 25
	plate II:	15 15 15 20 20 25 25 15	15 20 20 25 15 25 20 25	20 15 20 25 25 20 20 15
850	plate I :	10 25 15 10 05 15 05 15	15 20 15 10 15 15 15 15	15 05 15 15 15 05 10 10
	plate II:	10 25 15 20 10 15 25 15	15 15 15 15 15 10 25 15	15 15 15 20 05 15 15 10
855	plate I :	15 20 15 15 15 15 15 15	25 15 25 15 10 15 20 10	15 10 15 15 15 15 10 10
	plate II:	20 25 20 25 15 15 15 15	15 10 15 25 15 20 15 10	20 15 15 15 15 20 20 25
858	plate I :	20 15 20 15 05 15 25 15	15 15 20 15 15 25 20 15	25 20 15 10 25 25 15 05
	plate II:	15 15 20 20 05 05 25 15	15 25 15 15 15 20 20 20	15 15 15 15 20 25 10 15
830	plate I :	15 15 20 25 20 15 15 15	15 15 15 15 20 20 20 15	15 15 15 15 15 15 15 15
	plate II:	10 15 15 15 15 20 15 15	20 15 20 15 10 15 20 15	20 20 15 25 25 25 20 15

[1] Distance migrated as determined by the difference between the diameter of the spot of leucocytes after 24 hours incubation and before incubation, expressed as micrometer units, 8 spots per plate.

Table 2. Analysis of variance of data in Table 1.

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Square	F Value
(1) Chickens	5	745.23	149.05	4.32*
(2) Antigens	2	224.13	112.07	3.25
(3) Chickens x Antigens	10	344.62	34.46	0.89
(4) Plates/Chickens x Antigens	18	695.31	38.63	1.93
(5) Spots/Plates	252	5,034.38	19.98	
Total (N-1)	287	7,043.67		

* Significant at $\alpha = 0.05$

(1) Block effect

(2) Treatment effect

(3) Error term, Chickens by Antigens interaction

(4) Sampling error, Plates within Chickens by Antigens interaction

(5) Subsampling error

was significant among chickens, and ranged from 112.5 to 155 micrometer units.

The least square means were obtained for the leucocyte migration from chickens sensitized with BCG, CFA and control chickens, following sensitization, and in vitro testing of leucocyte migration with the antigens OT and B-24. The least square means represent weighted average migration of leucocytes adjusted for missing data and unequal sample sizes. The least square means are given in Table 3.

On the basis that inhibition of migration in the presence of the antigen was used to detect in vitro sensitivity, the results indicated the following: (1) OT at both concentrations tested inhibited migration of leucocytes from sensitized as well as control chickens. (2) B-24 enhanced the migration of leucocytes from the control group and CFA sensitized group, resulting in migration as far or further than leucocytes with no antigen. (3) B-24 inhibited in vitro migration of leucocytes from the BCG sensitized group. (4) B-24 appeared to eliminate the possibility of a false positive migration inhibition. The data were further analyzed to determine the contribution of chickens, sensitization, antigen, plates, and spots in affecting significantly the migration of leucocytes. The statistical model is a split plot, random model design with sampling and subsampling. Results of the analysis are in Table 4.

Table 3. Leucocyte migration by three groups of chickens with antigens. Migration summarized as least square means.

Group	Number of chickens/group	(4) Least square means of migration			
		No antigen	25 µg OT	50 µg OT	B-24
(1) Control	4	15.22	15.04	12.35	27.81
(2) CFA	8	29.54	24.98	23.15	30.57
(3) BCG	8	21.62	16.47	16.68	13.71

(1) Nonsensitized

(2) Complete Freund's adjuvant in emulsion with PBS, injected subcutaneously

(3) Bacille Calmette-Guerin in emulsion with IFA, injected subcutaneously

(4) MIF test, 2 months after sensitization

Table 4. Analysis of variance for data summarized in Table 3 and analyzed as a split plot random model.

	Source of Variation	Degrees of Freedom	Sums of Squares	Mean Squares	F Value
	Sensitizations	2	34,528.12	17,264.12	6.19*
(1)	Chickens/Sensitizations	17	47,433.99	2,790.23	
	Antigens	3	7,862.66	2,620.87	11.22*
	Sensitizations x Antigens	6	5,464.30	910.72	3.90*
	Antigens x Chickens/ Sensitizations	51	9,610.69	188.44	0.81
(2)	Plates/Antigens x Chickens/ Sensitization	76	17,745.83	233.50	5.80*
(3)	Spots/Plate	1090	43,912.50	40.29	
	Total	1245	166,558.09		

* Significant $\alpha = 0.05$

(1) Error A

(2) In this analysis plates/sensitization, chickens, antigens is used as the error B term and is also the sampling error.

(3) Subsampling

The error term usually used to construct the test F ratio is the value represented by antigen by chickens within sensitization effect (Steel and Torrie, 1960). However, because there were significant variations between chickens, and the chicken effect was confounded with sensitization, the error term used in this analysis was plates within sensitization, chickens, antigen. It is a more conservative statistic and less likely to result in an inflated F value.

Sensitization and antigen significantly affected leucocyte migration as shown by the analysis in Table 4. Because sensitization itself affected migration significantly, and there was a sensitization by antigen interaction, the independent effect of antigen on migration could not validly be compared among sensitization groups. Therefore, supplementary testing was conducted to determine whether antigen inhibited leucocyte migration in the respective sensitization groups. The supplementary statistical analyses comparing least square migration means of leucocytes incubated with the antigens are given in Table 5 by sensitization groups. The analysis in Table 4 indicates that the variability of migration of individual spots was great, and that the leucocyte migration was significantly different between duplicate plates.

The results summarized in Table 5 indicate that both concentrations of OT inhibited migration of leucocytes from sensitized and control chickens, and did not discriminate

Table 5. Comparison of leucocyte migration means of Control, CFA sensitized and BCG sensitized chickens with antigen. Supplementary testing for data analyzed in Table 4.

Sensitization	Migration comparison	Test ratio	Degrees of Freedom	T-statistic testing for inhibition
(1) Control	no antigen vs. 25 µg OT	$\frac{3.125}{1.285}$	(1,51)	2.43*
	no antigen vs. 50 µg OT	$\frac{5.156}{1.285}$	(1,51)	4.01*
	no antigen vs. B-24	$\frac{-8.359}{1.285}$	(1,51)	-6.51
(2) CFA	no antigen vs. 25 µg OT	$\frac{2.904}{0.974}$	(1,51)	2.98*
	no antigen vs. 50 µg OT	$\frac{6.497}{0.974}$	(1,51)	6.86*
	no antigen vs. B-24	$\frac{0.990}{0.974}$	(1,51)	1.02
(3) BCG	no antigen vs. 25 µg OT	$\frac{2.344}{0.909}$	(1,51)	2.58*
	no antigen vs. 50 µg OT	$\frac{6.016}{0.937}$	(1,51)	6.42*
	no antigen vs. B-24	$\frac{4.648}{0.909}$	(1,51)	5.11*

* Significant, $\alpha = 0.05$
T statistic tests for inhibition of leucocyte migration by antigen and therefore is used as a one-tailed test

(1) See footnote Table 3; (2) See footnote Table 3; (3) See footnote Table 3)

immunologic sensitivity from nonsensitivity on the basis of leucocyte migration at the antigen concentrations tested. The results of a pilot study were no inhibition of leucocyte migration by 10 and 20 µg OT and leucocyte mortality at concentrations of 100 µg OT. In contrast, B-24 did not inhibit migration of leucocytes from control chickens or CFA sensitized chickens. Rather, it enhanced migration in controls and inhibited migration in the BCG group. The activity of B-24 *in vitro* was immunologically more specific than OT. This selective inhibition in migration of leucocytes by B-24 from BCG sensitized chickens but not from control or CFA chickens also contributed to the significant interaction between sensitization and antigen, as given in Table 4.

As previously reported (Fauser *et al.*, 1973c), the cells attaching to the petri dish were approximately 40% mononuclear and 60% polymorphonuclear leucocytes when stained with Wrights stain. The morphology of leucocytes at the end of 24 hours of incubation was altered by cytoplasmic vacuoles so that a differential analysis of cell type was not possible.

The results of skin reactions, antibody determinations and pathologic involvement of organs and tissues are summarized in Table 6. The control group remained negative for antibody, skin reactions and pathologic reactions. Both sensitized groups had positive skin reactions to OT: 5/8

Table 6. Skin reactions, antibody responses and pathologic reactions of 3 groups of chickens in Experiment I.

Group	Chicken	(4) Skin reaction with OT	(4) Skin reaction with B-24	(5) Precipitating antibody to PPD	(6) Lesions and/or acid fast organisms
(1) Control	827	-	-	-	-
	No band	-	-	-	-
	829	-	-	-	-
	823	-	-	-	-
Sub-total		0/4	0/4	0/4	0/4
(2) CFA	851	+	+	+	+
	850	+	-	+	+
	855	+	-	+	+
	856	+	+	+	+
	843	-	+	+	+
	844	(7)+	+	+	+
	857	(7)-	+	+	+
	858	-	-	+	+
Sub-total		5/8	5/8	8/8	8/8
(3) BCG	838	-	+	+	+
	848	+	+	+	-
	839	+	+	+	+
	842	+	+	+	+
	830	+	+	+	+
	833	+	+	+	+
	834	+	+	+	+
	835	-	+	+	+
Sub-total		6/8	8/8	8/8	7/8

(1) See footnote Table 3.

(2) See footnote Table 3.

(3) See footnote Table 3.

(4) Skin reactions were graded positive if a minimum of a two-fold increase in wattle thickness as compared with the uninoculated wattle was attained.

(5) A two-fold concentration (0.01 mg protein) of second strength purified protein derivative (PPD, Parke-Davis, Detroit, Mich.) was used in double diffusion in agar coated slides. All chickens were antibody negative prior to sensitization.

(6) Granulomatous and/or caseous lesions, gross, and/or microscopic, some with acid fast organisms were recorded as (+) positive. A negative (-) indicates neither lesions nor acid fast organisms were found.

(7) + = positive

- = negative

CFA and 6/8 BCG. More BCG sensitized chickens than CFA chickens responded with skin reactions to B-24: 8/8 as compared to 5/8. Precipitating antibody to PPD was present in sera from all sensitized chickens. Lesions, some with acid fast organisms, were found in 8/8 CFA sensitized and 7/8 BCG sensitized chickens. All BCG injected chickens had caseous granulomas of the serosa of the gizzard. Lesions in chickens injected with CFA were either granulomatous or granulomatous with caseation necrosis. No acid fast organisms were recovered by bacteriologic culture.

B. Experiment II

Leucocyte migration was tested in nine chickens to determine whether the antigens OT and crude MDV inhibited migration prior to sensitization. The statistical model is a randomized complete block design with sampling and subsampling. The results are given in Table 7.

As in Experiment I, the individual variation in leucocyte migration among chickens was statistically significant. The presence of antigen in the culture medium had a significant effect on leucocyte migration and the effect of antigen was similar for leucocytes of all chickens tested. This is reflected by no significant interaction between chickens and antigens. Leucocyte migration of spots in duplicate plates under identical culture conditions were significantly different.

Table 7. Analysis of variance of migration of leucocytes with and without the antigens crude GA and OT from control chickens.

	Source of Variation	Degrees of Freedom	Sums of Squares	Mean Square	F Value
(1)	Chickens	8	39,302.08	4,912.76	33.05*
(2)	Antigens	2	3,044.79	1,522.40	10.24*
(3)	Chickens x Antigens	16	2,378.13	148.63	1.58
(4)	Plates/Chickens x Antigens	27	2,543.75	94.21	2.95*
(5)	Spots/Plate	378	12,062.50	31.91	
	Total	431	59,331.25		

* Significant at $\alpha = 0.05$

(1) - (5) See footnotes, Table 2.

The mean migrations by individual chicken's leucocytes with and without antigen are summarized in Table 8. The table presents mean migration in micrometer units of all leucocyte spots from nine control chickens with no antigen, OT, and crude GA antigen. The variation among chickens was reflected in the range of mean (arithmetic) migration for all spots with each of three antigens: (1) with no antigen the range of mean migration was 13.4 to 43.4 micrometer units, (2) with 50 µg OT 7.2 to 36.6 and (3) with crude GA antigen 8.1 to 36.9. The effect of antigen on migration was reflected in the mean migration of leucocytes from all chickens with no antigen as a mean of 23.4, 50 µg OT a mean of 19.0 and crude GA a mean of 17.0. Inspection of these mean values with the Wilcoxon matched-pairs signed-ranks test (Siegel, 1956) showed that each antigen significantly ($\alpha = 0.02$) inhibited mean migration of leucocytes as compared with no antigen. Incubation of leucocytes with an extract prepared from the same cells used to culture the GA strain of MDV resulted in no inhibition of leucocyte migration.

The results of the statistical analysis of migration of leucocytes from the same chickens, but following sensitization are given in Table 9. No significant effect of sensitization or antigen *per se* was detected. However, there was a significant antigen sensitization interaction, which is tabulated as least square means in Table 10. As in the control (presensitization) analysis, antigen *per se* did not

Table 8. Mean migration in micrometer units by all leucocyte spots from 9 control chickens with no antigen, OT, and crude GA.

Chicken number	No antigen	50 µg OT	Crude GA
1594	41.6	36.6	30.3
1595	43.4	32.5	36.9
1597	16.6	17.5	10.9
1604	24.7	19.1	15.0
1606	15.6	14.1	10.6
1607	23.8	18.4	13.4
1612	15.0	9.1	16.9
1613	16.3	16.6	10.9
1614	13.4	7.2	8.1
$\bar{x}$:	23.4	19.0	17.0

Table 9. Analysis of variance of *in vitro* migration of leucocytes from control and MDV sensitized chickens analyzed with the least square routine as a split plot random model.

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Square	F Value
Sensitizations	2	3,789.39	1,894.70	2.94
(1) Chickens/Sensitizations	6	3,867.10	644.52	
Antigens	2	391.91	195.96	3.14
Antigens x Sensitizations	4	1,946.89	486.72	7.79*
(2) Antigen x Chickens/Sensitization	12	1,853.70	154.48	2.47
(3) Plates/Sensitization, Chickens x Antigens	26	1,624.48	62.48	1.33
(4) Spots/Plate	367	17,235.57	46.96	
Total	419	30,709.04		

* Significant at $\alpha = 0.05$

(1) Error A

(2) Error B usually used

(3) Sampling and error B

(4) Subsampling

Table 10. Antigen by sensitization interaction of data on Table 9 represented by least square means migration with and without antigen.

Group	Number of chicken/group	Least square means of migration		
		No antigen	50 µg OT	Crude GA
Control	3	11.98	13.63	15.80
(1) GA	3	13.32	13.02	8.23
(2) GA + CFA	3	22.71	15.83	17.29

(1) GA strain MDV injected and/or chickens in contact exposure to GA strain MDV injected chickens

(2) GA strain MDV injected in emulsion with complete Freund's adjuvant

significantly affect migration from one chicken to the next. The analysis also indicated that no significant differences could be detected between duplicate plates.

The least square means tabulated in Table 10 indicate the control group had no detectable inhibition of leucocyte migration with either OT or crude GA antigen. The GA group was inhibited significantly by GA antigen and not by OT and the GA+CFA group was inhibted by both OT and crude GA.

The results of leucocyte migration of MDV infected and control chickens with A-antigen in the culture medium are given in Table 11. In this experiment, one plate with seven spots was prepared for each culture condition being tested.

A-antigen inhibited radial leucocyte migration in cultures from all MDV infected chickens by 16 to 66%, with a mean of 44.4%. It enhanced migration of leucocytes from the normal chickens by a mean of 9.7%.

Results of skin tests and antibody determinations are given in Table 12. Control chickens had no skin reactions with OT, crude GA and A-antigen. They had no detectable antibody to MDV or PPD. Sensitization of chickens with GA-MDV in CFA induced sensitivity to tuberculin as detected by skin tests to OT and antibody responses to PPD, but did not induce sensitivity to GA-MDV as determined by antibody and skin reactions. Chickens sensitized with viable GA-MDV and those in contact exposure to MD had positive skin reactions to crude GA and A-antigens and had fluorescent antibody to

Table 11. Arithmetic mean migration of leucocytes from control and MDV chickens with no antigen and A-antigen.

Chicken number	Treatment	Average migration of 7 spots		(1) Average percent migration inhibition
		No antigen	A-antigen	
1612	Control	41.1	45.7	-11.3
1613	Control	43.2	43.6	- 0.8
1614	Control	31.1	36.4	-17.1
1594	MDV	23.2	7.9	66.1
1595	MDV	40.4	21.4	46.9
1596	MDV	45.0	27.9	48.1
1597	MDV	22.1	22.1	16.3

(1) Percent inhibition = $\left[\frac{\text{distance migrated with antigen}}{\text{distance migrated without antigen}}\right]$ x 100

Table 12. Skin reactions and antibody responses of chickens in Experiment II.

Group	Chicken	(4) Skin Reaction to OT	(4) Skin Reaction to Crude GA	(4) Skin Reaction to A-antigen	(8) MDV antibody	(5) Antibody to PPD
(1) Control	1611	-	-	-	-	-
	1612	-	-	-	-	-
	1613	-	-	-	-	-
	1614	-	-	-	-	-
(2) GA in CFA	1604	(7)+	(7)-	-	-	+
	1606	+	-	-	-	+
	1607	+	-	-	-	+
	1608	+	-	-	-	+
	1609	+	-	-	-	-
(3) GA	1594	-	+	+	+	(6)ND
	1595	-	+	+	+	ND
	1596	-	+	ND	ND	ND
	1597	-	+	+	+	ND

(1) Nonsensitized
(2) MDV strain GA in complete Freund's adjuvant
(3) MDV strain GA injected and/or in contact exposure to MDV strain Ga injected chickens
(4) See footnote Table 6. Skin reaction to crude GA antigen resulted in thickening of wattle at 36 hours diminishing to normal by 48 hours. Skin reaction to A-antigen resulted in swelling and induration at 36 hours diminishing to normal by 48 hours.
(5) See footnote Table 6
(6) Not determined
(7) See footnote Table 6
(8) Fluorescent antibody test

MDV. With crude GA antigen, the thickening of the wattle commenced at 25 hours and had mostly disappeared by 48 hours. With A-antigen a small amount of swelling and induration was detected at 36 hours, but had disappeared by 48 hours.

At necropsy none of the chickens had lesions. All MDV infected chickens were emaciated. No neural lesions were found on histopathologic examination of those chickens injected with and/or exposed to MDV.

C. Experiment III

The least square mean weight in grams of 19 day chicken embryo spleens from embryos injected at 14 days embryonation with leucocytes from intact, bursectomized, and thymectomized donors is shown in Table 13.

The variability of spleen weights was great among those embryos receiving leucocytes from donors belonging to different treatment groups. The least square spleen weights of embryos injected with leucocytes from the three treatment groups were as follows: (1) Intact 0.1059 grams, (2) Bursectomized 0.1101 grams, (3) Thymectomized 0.1527 grams. The mean spleen weights of the controls were as follows: (1) Maternal leucocytes 0.0442, (2) Tissue culture medium 0.0142, and (3) Uninoculated 0.0141. The recorded spleen weight means in some cases are from fewer than 10 embryos

Table 13. Least square mean spleen weights in grams of 19-day old embryos injected at 14 days embryonation with leucocytes from intact, bursectomized and thymectomized donors and mean spleen weights of control spleens.

Intact	Bursectomized	Thymectomized	Control
(1) 0.1093	0.1342	0.1187	(2) 0.0442 (10)
0.1687	0.0748	0.1125	(3) 0.0141 (9)
0.1214	(1) 0.1844	0.1540	(3) 0.0143 (10)
0.0688	0.1241	0.1244	(4) 0.0141 (9)
0.1331	0.0978	0.0911	$\bar{x}$ 0.0217
0.1381	0.1206	0.1251	
0.0902	0.0694	0.1347	
0.1692	0.1689	$\bar{x}$ 0.1527	
0.1060	0.1739		
0.1367	0.0808		
0.1135	$\bar{x}$ 0.1101		
0.1199			
$\bar{x}$ 0.1059			

(1) Least square mean spleen weights from embryos injected with leucocytes from 1 donor chicken, less than 10 embryos survived to 19 days embryonation.

(2) Spleen weights from embryos injected with leucocytes from one of the dams, number in ( ) is number of embryos surviving to 19 days embryonation.

(3) Mean spleen weights of embryos injected with tissue culture medium.

(4) Mean spleen weights of uninoculated embryos.

due to embryo mortalities. In determining if thymectomy or bursectomy of the donors affected the GVH capability of their leucocytes the data were analyzed using regression analysis, a weighting procedure to compensate for unequal numbers of replicates and missing data. The analysis is shown in Table 14. The greatest splenomegaly was elicited by the leucocytes from the thymectomized group of donors. All donor chickens were negative for MDV antibody with the indirect fluorescent antibody test. Because there was increased instead of the expected decreased splenomegaly the experiment was repeated. To reduce the variability of spleen weight response to the individual donor leucocytes by recipient embryos, five each of sublines 1 and 4 were injected and recorded separately as recipients of each donor leucocyte inoculum. Also, the bursectomized donor group was omitted. The least square means of embryo spleen weights resulting from injection with leucocytes, are presented in Table 15. All donors had no detectable MDV antibody. With one exception, there was greater splenomegaly in embryos of subline 1 than subline 4 injected with leucocytes from the same donor. This is significant in indicating that both sublines responded in a parallel manner to the injection of leucocytes from the same donor. Thymectomized donor leucocytes elicited greater splenomegaly 0.1249 grams, than intact donors leucocytes, 0.0667 grams. The mean spleen weights of embryos inoculated with the leucocytes

Table 14. Analysis of variance for data summarized in Table 13.

Source of Variation	Degrees of Freedom	Mean Square	F Value
Surgery	2	5,298,146.8931	20.5716*
Chickens	26	1,036,198.7629	4.0233*
Error	254	257,546.1480	
Total	282		

*Significant $\alpha = 0.05$

Table 15. Least square means of spleen weight in grams by embryos of two sublines of line 15 injected with leucocytes from intact and thymectomized donors.

Intact		Thymectomized	
Subline 1	Subline 4	Subline 1	Subline 4
0.1089	0.0827	0.1691	0.0224
(1) 0.0711	(1) 0.1205	0.1168	0.0748
0.1310	0.0606	0.0962	0.0953
0.1274	0.0642	0.1430	0.0486
0.1196	0.0720	0.1292	0.0624
0.1277	0.0638	0.1388	0.0527
0.0667		0.1249	

(1) Only case where least square mean of spleen weight of subline 1 was less than that of subline 4.

of the same subline and the heterologous subline, embryos inoculated with the culture medium used as the diluent for donor leucocytes, and uninoculated embryo controls are given in Table 16. In all controls the mean spleen weights of line 15 subline 1 were greater than those of subline 4. The embryos receiving syngeneic donor leucocytes had slight splenomegaly but less than embryos which received leucocytes from the other subline.

The analysis of variance of the data summarized in Table 15 is given in Table 17. The greater splenomegaly of embryos inoculated with leucocytes from thymectomized chickens was significant. Donor chickens differed significantly in the capacity of their leucocytes to induce splenomegaly. The differences in splenomegaly by each of the sublines is significant.

The analysis of the results obtained from the study to determine whether thymectomized sensitized chickens have impaired MIF production in vitro is summarized in Table 18. There were no missing data, and all sample sizes were equal.

The analysis indicates that sensitization with BCG as nested in time (times 1 and 2 are prior to sensitization and times 3 and 4 are after sensitization) does not, in and of itself, detectably influence leucocyte migration in vitro. This also indicates the validity of repeated measurements in time. The response of leucocyte migration in vitro does not appear to change appreciably during the time frame measured.

Table 16. Mean spleen weights of controls for data on Table 15.

Donor	Recipient	Spleen Weight
(1) 15 subline 4	(1) 15 subline 1	(2) 0.1122 (5)
15 subline 1	15 subline 4	0.0930 (4)
15 subline 1	15 subline 1	0.0281 (5)
15 subline 4	15 subline 4	0.0198 (5)
F10-199+15% SPF serum	15 subline 1	0.0169 (5)
F10-199+15% SPF serum	15 subline 4	0.0168 (5)
no donor	15 subline 1	0.0171 (5)
no donor	15 subline 4	0.0162 (5)

(1) Line 15

(2) Number in ( ) is number of embryos surviving to day 19 of embryonation.

Table 17. Analysis of variance for data summarized in Table 15.

Source of Variation	Degrees of Freedom	Mean Square	F Value
Surgery	1	8,713,870.7056	139.9806*
Chickens	10	1,708,627.6925	27.4476*
Line (Chicken x Surgery)	12	1,135,994.1719	18.2487*
Error	82	62,250.5764	
Total	105		

*Significant $\alpha = 0.05$

Table 18. Analysis of variance of surgery, sensitization, and antigen in leucocyte migration *in vitro*.

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Square	(2) F Value
(1) Time	3	7,188.77	2,396.26	1.43
Surgery	2	32,580.88	16,290.44	3.39
Time x Surgery	6	14,932.44	2,488.74	1.49
Chickens/Surgery	18	86,528.68	4,807.15	
Time x Chickens/Surgery (Error A)	54	90,257.70	1,671.44	
Antigen	1	85.71	85.71	0.29
Antigen x Time	3	1,763.47	587.82	3.81*
Antigen x Surgery	2	88.62	44.31	0.29
Antigen x Time x Surgery	6	2,231.85	371.98	2.41*
Antigen x Chickens/Surgery	18	5,298.33	294.35	1.91*
Antigen x Time x Chickens/ Surgery	54	8,324.22	154.15	1.04
Plates/Time x Surgery x Chickens x Antigen	168	24,962.50	148.59	2.63*
Spots/plate	2,352	132,968.75	56.53	
Total	2,687	407,211.92		

* Significant at $\alpha = 0.05$

(1) Times 1 and 2 were presensitization leucocyte migration in presence and absence of B-24 and times 3 and 4 were leucocyte migrations in presence and absence of B-24 after sensitization.

(2) Variances were homogeneous, Bartletts test.

The surgical manipulation of thymectomy or bursectomy does not detectably influence migration; however, there was a suggestive effect of surgery ($\alpha = 0.055$) considering the mean migration of all leucocyte spots for all 4 times of measurement were as follows: (1) Intact, 36.0; (2) Bursectomized, 41.9; and (3) Thymectomized, 33.6 micrometer units. There was also no interaction of time (sensitization) with surgery so the effect on migration by surgery only remained similar for all groups.

Antigen, in this case B-24, did not detectably affect migration at a significant level. The overall means for migration without antigen, over all time periods and surgery groups was 37.4 micrometer units and with B-24, 37.0 micrometer units.

There is a statistically significant interaction between antigen and time, and to represent this interaction, the means are given in micrometer units in Table 19. Inspection of the means of leucocyte spots, regardless of individual chicken and surgery effects indicates leucocyte migration was greater with B-24 than with no antigen when assayed prior to sensitization. B-24 inhibited migration after sensitization.

The absence of a significant interaction of antigen and surgery indicated that the presence or absence of B-24 was associated with similar migration for intact, thymectomized and bursectomized chickens.

Table 19. Migration means for antigen by time interaction (1) Table 18.

Incubation with	Presensitization		Postsensitization	
	Time 1	Time 2	Time 3	Time 4
No antigen	33.6	38.3	37.8	39.8
B-24	35.6	38.6	36.3	37.7

(1) At both presensitization times, there was no migration inhibition, as summarized by means, rather enhancement by the antigen B-24. At both postsensitization times, the means indicated inhibition of migration by the antigen B-24.

The antigen by time by surgery interaction is most noteworthy, and of greatest interest in determining whether migration inhibition by the sensitizing antigen in sensitized thymectomized chickens was diminished or absent as predicted. A summary, using group means, of the data comprising the interaction is given in Table 20.

The parallel response trend of the normal and bursectomized groups is evident. During both presensitization assays, B-24 enhanced migration in the intact and bursectomized groups, and inhibited migration during both postsensitization assays. In contrast, the thymectomized group means indicate possible enhancement during the first presensitization assay and inhibition during the second presensitization assay. Following sensitization, there was inhibition during the first postsensitization assay and enhancement during the second. The response of the thymectomized group did not parallel that of the intact and bursectomized groups.

In this experiment there was no significant interaction of antigen by time by chickens within respective surgery, meaning that respective chickens varied in their leucocyte migration with and without antigen during the four time frames sampled in a similar manner. When the data were expressed in terms of mean percent increase in migration inhibition of the postsensitization versus presensitization group levels, the intact group was characterized by an

Table 20. Migration means in micrometer units for antigen by time by surgery interaction.[1]

Surgery Group	Incubation with	Presensitization		Postsensitization	
		Time 1	Time 2	Time 3	Time 4
Intact	no antigen	28.5	35.8	39.6	39.9
	B-24	31.3	37.8	37.3	38.1
Bursectomized	no antigen	41.3	45.2	37.5	44.9
	B-24	44.1	46.1	37.4	39.0
Thymectomized	no antigen	31.1	33.8	36.3	34.7
	B-24	31.4	31.9	34.1	36.0

(1) Both intact and bursectomized groups were characterized by means which indicated no migration inhibition by B-24 as compared to no antigen during the nonsensitized time periods and by inhibition during the time periods following sensitization. In this regard the response of leucocytes from intact and bursectomized groups was parallel. In contrast, the thymectomized group was characterized by both apparent enhancement and inhibition by B-24 as compared with no antigen during the two pre- and postsensitization time periods. The migration response to no antigen and B-24 of the thymectomized group did not parallel that of the intact and bursectomized groups.

increase of 11.6%, the bursectomized 10.4% and thymectomized 1.3%. Duplicate plates were significantly different in the migration values of their leucocyte spots in this experiment.

The skin reactions, antibody responses and incidence of gross and/or microscopic tuberculosis-like lesions of all adult chickens of Experiment III are summarized in Table 21. Skin tests with B-24 were negative in all chickens prior to sensitization and no precipitating antibody to PPD or precipitating and/or fluorescent antibody to HVT were detected. Following sensitization B-24 elicited delayed skin reactions in 6/7 intact, 5/7 bursectomized and 3/7 thymectomized chickens. Consequently, whether the positive reactions at 48 hours of all chickens in the thymectomized and control groups was a true delayed or persistent Arthus reaction cannot be resolved. Antibody to PPD and HVT was detected in all intact chicken and in none of those bursectomized. Five of seven thymectomized chickens had detectable antibody with PPD and all had antibody to HVT. Gross and/or microscopic lesions, some with acid fast organisms, were found in 6/7 intact, 4/5 bursectomized, and 5/7 thymectomized chickens.

Some of the thymectomized chickens had thymic remnants. These consisted of small fragments at the most caudal location of the thymus. In no case were complete lobes

remaining. Completeness of bursectomy could not be detected, because in all chickens the bursa had involuted.

Table 21. Skin reactions, antibody response, and lesions of adult chickens in Experiment III.

Group	Chicken number	(1) Skin Reactions B-24		OT		Antibody		(5) Lesions
		Delayed	(2) Arthus	Delayed		(3) PPD	(4) HVT	
Intact	8926	+	+	(8)	?	+	+	+
	8927	+	-		+	+	+	+
	8918	(6) +	-		+	+	+	+
	8920	(6) -	+		?	+	+	-
	8921	+	-		+	+	+	+
	8925	+	-		+	+	+	+
	8928	+	+		?	+	+	+
Group subtotals		6/7	3/7		7/7	7/7	7/7	6/7
Bursectomized	8906	-	(7) ND		ND	ND	ND	ND
	8907	+	-		+	-	-	+
	8908	+	+		+	-	-	+
	8910	+	-		+	-	-	+
	8911	-	-		+	-	-	-
	8912	+	-		+	-	-	-
	8916	+	ND		ND	ND	ND	ND
Group subtotals		5/7	1/5		5/5	0/5	0/5	4/5
Thymectomized	8894	+	+		?	+	+	+
	8895	-	+		?	-	+	-
	8897	-	+		?	+	+	+
	8898	+	+		?	+	+	+
	8900	-	+		?	+	+	+
	8902	-	-		?	-	+	-
	8903	+	+		?	+	+	+
Group subtotals		3/7	6/7		7/7	5/7	7/7	5/7

(1) Skin reactions were graded positive if a minimum of a two-fold increase in wattle thickness as compared with the uninjected wattle was attained. Skin tests with B-24 prior to sensitization were negative in all chickens.

(2) Skin reaction detectable as early as 2 hours after testing whether reaction at 48 hours was true delayed or Arthus is not known (?)

(3) A two-fold concentration (0.01 mg protein) of second strength purified protein derivative (PPD, Parke-Davis, Detroit, Mich.) was used in double diffusion in agar coated slides. All chickens were antibody negative prior to sensitization.

(4) Fluorescent and precipitating antibody. Antibody response prior to sensitization was negative in all chickens.

(5) Granulomatous and/or caseous lesions, gross, and/or microscopic, some with acid fast organisms were recorded as (+) positive. A negative (-) indicates neither lesions nor acid fast organisms were found.

(6) + = positive
- = negative

(7) Not determined because these 2 chickens drowned.

(8) ? = questionable.

DISCUSSION

Factors Influencing In Vitro Leucocyte Migration

Some of the designated variables in the experimental design influenced leucocyte migration of control and sensitized chickens. Thus, the variables relating to the experimental procedure and biologic assumptions of the MIF test and the significance of the results of the various hypothesis testing experimental objectives are discussed herein.

The primary objective was to determine whether an MIF test using chicken blood leucocytes could be developed. Prior to testing whether leucocytes from sensitive chickens were inhibited by antigen during *in vitro* migration, controls necessary for biologic reliability had to be tested. The innate variability in migration by individual control chickens in Experiments I and II (Tables 2 and 7), indicated that a standardized value in micrometer units to differentiate migration from inhibition was not possible. Had it been, the design of the MIF test as used with chicken leucocytes could have been simplified to require no control (nonantigen containing) plates and statistical analyses of data would have been easier by the application of nonparametric statistics (Siegel, 1956). The significant

individual chicken variation in leucocyte migration led to the use of the alternate experimental error for the test F ratio rather than the one usually employed (Tables 4 and 9). This was done to minimize bias which would otherwise be introduced by use of the sampling error which was itself significant as a variable.

Statistically, the presence of antigen in the culture medium could significantly affect leucocyte migration in control chickens (Table 7). If migration or inhibition by antigen were evaluated by eyeball comparison with that of nonantigen containing plates (Tables 3 and 8) the antigen effect was significant. It became an objective to eliminate the inhibition by antigen of leucocyte migration of control chickens, rather than identify its cause. The presence of 0.05% phenol, as a preservative, in OT may have inhibited leucocytes migration without killing leucocytes. Leucocytes inhibited in migration remained viable, on the basis of Trypan blue exclusion.

The apparent inhibition in migration by crude GA antigen (Table 8) may be due to the possible presence of hydrolytic enzymes released during extraction of viral antigenic material from chick embryo fibroblasts; however, identically processed non-MDV infected chick embryo fibroblast extracts did not inhibit leucocyte migration. The increase in leucocyte migration of control chickens by B-24 (Table 3) and

A-antigen (Table 11) indicated that these antigen preparations did not inhibit leucocyte migration of control chickens and complied with the criteria established by George and Vaughn (1962) and David *et al*. (1964, 1964a) that antigen not inhibit migration of leucocytes from nonsensitized guinea pigs, only from sensitized ones. Because the antigens OT and crude GA inhibited migration of leucocytes in control chickens, their use in developing an MIF test in chickens was not justified. B-24 and A-antigen *in vitro* caused no inhibition, rather enhancement, of leucocyte migration by control chickens, thus eliminating the false positive result. Both B-24 and A-antigen were prepared in other laboratories for independent studies and relatively small quantities, 3 ml B-24 and 4 ml A-antigen, were available for *in vitro* chicken leucocytes migration studies. Therefore, the smallest concentrations of these antigens that gave immunologically detectable responses were used *in vivo* and *in vitro*. Presumably they were just at threshold levels or concentrations that could be diluted with reasonably little technical error. B-24 was used at no less than 2 µg/ml *in vitro* to facilitate accuracy in dilution and A-antigen was used at a 1/8 dilution because this concentration detected precipitating MDV antibody. The sensitivity of migration to varying antigen preparations by lymphocytes and macrophages cultured *in vitro* has been reported previously (UN-WHO, 1973).

In addition to individual chicken and antigen influences on leucocyte migration, sensitization was found to influence migration in Experiment I (Table 4). In Experiment I the choice of substance, either CFA or BCG, for inducing sensitivity influenced leucocyte migration significantly. To illustrate this influence, the least square means for control and sensitized groups were calculated (Table 3). Migration of leucocytes of the CFA group was greater than control and BCG groups under all four *in vitro* culture conditions. The reason for the apparent increase in migration of leucocytes from CFA chickens is not known. The composition of spots of leucocytes was similar for all groups, approximately 40% mononuclear and 60% polymorphonuclear (Fauser *et al.*, 1973a). Whole blood obtained from both sensitized groups consistently had a greater volume of the buffy coat per 10 ml blood. In Experiment II there was also a volume increase in the buffy coat of MDV sensitized chickens, as previously reported (Hudson and Payne, 1972) but no statistically detectable increase in migration due to sensitization alone. The increase in the buffy coat was not thought to directly explain increased migration by CFA sensitization. The increase in migration of leucocytes from CFA sensitized chickens merits further study. Perhaps it reflects a nonspecific leucocyte or macrophage activation as evidenced in heightened physiologic activity. Because of the effect of CFA in increasing the leucocyte migration under

all culture conditions in Experiment I, BCG, the alternate sensitization, was chosen for inducing delayed sensitivity in Experiment III. Sensitization in Experiment II with MDV and/or CFA did not detectably influence leucocyte migration. If sensitization alone or antigen alone did not inhibit leucocyte migration, then the hypothesis that the appropriate antigen inhibits migration in sensitive animals could be tested directly. The results of the analysis of in vitro migration in Experiment III (Table 18) indicated that sensitization, with BCG as confounded with time, did not detectably enhance leucocyte migration. Albeit the power of the error term (error a) used to test time effect in Experiment III was reduced by nature of the split plot design.

Because sensitization alone influenced migration in Experiment I (Table 4) the data did not justify a statistically valid comparison of effect of antigen on leucocyte migration inhibition to be made across sensitization groups even though it was of biological interest to do so. It was hoped to compare statistically, migration values resulting from incubation of leucocytes from control, BCG sensitized and CFA groups to determine if one could discriminate among BCG and CFA sensitization and controls on the basis of migration or inhibition. Instead, the statistical significance of antigen on leucocyte migration was confined to and tested independently within each sensitization group. Only observational interpretation and description can be validly

made of antigen among sensitization groups as a result of the statistically significant effect of sensitization itself on migration.

Another designated test variable was the reliability of migration or inhibition between duplicate plates. Spots of leucocytes on duplicate plates occasionally migrated different distances, these were statistically significant differences. There was no apparent correlation between sensitization effect and plate differences. In Experiment I (Table 2) duplicate plates of leucocytes from nonsensitized chickens were not detectably different in migration, but following sensitization with BCG or CFA, migration between duplicate plates was significantly different (Table 4). In contrast, plate differences were detectable in Experiment II prior to sensitization (Table 7) and not after sensitization with Mycobacteria and/or MDV (Table 9). Plate differences were also detected in Experiment III (Table 18).

Although the reason for plate differences cannot be readily explained, the determination that duplicate plates differed significantly and the partitioning of this effect from the error term contributed to the reliability of the error term. Had the plate effect been unknown, and not partitioned from the experimental error, the denominator of the test statistic (F-ratio) would have been smaller in Tables 4 and 9. This would have resulted in claims of more test variables being statistically significant than actually

were reported. Testing more than 2 plates per chicken per antigen may have determined that the differences between duplicate plates represented significant sampling error rather than a true plate difference.

Large enough quantities of B-24 and A-antigen were not available to prepare additional plates containing 2 ml tissue culture medium with antigen for leucocytes of all chickens within the respective experiments. Because leucocytes were more readily available than antigen, the maximum number of spots of leucocytes were attached to 2 plates rather than distributed among 4, for example.

When B-24 and A-antigen were used in vitro with leucocytes from BCG or MDV infected chickens, the MIF test resulted in predictable migration or inhibition. The enhancement in migration by leucocytes incubated with B-24 and obtained from control chickens facilitates the rigid and reliable test to determine nonsensitivity. Since B-24 inhibited migration of the BCG sensitized group, and not the controls, or the CFA group, its use in vitro appears to be justified. Before concluding that it can discriminate among a wider variety of Mycobacterial and/or mycotic sensitivities, more sensitization groups would have to be tested. Sensitization with CFA was chosen in Experiment I because it is widely used experimentally to induce delayed sensitivity and it is also used to stimulate a humoral immune response by incorporation of additional antigens in CFA. It would be

of theoretic as well as practical significance to be able to demonstrate the simultaneous presence of T cell sensitivity with the humoral response in cases where CFA is employed. Had OT not inhibited migration by the leucocytes from chickens in the control group, it was hoped OT would demonstrate in vitro the delayed sensitivity induced by CFA. For in vitro studies in which the simultaneous T and B cell responses need to be tested, BCG can be incorporated with IFA, as was done in Experiment III. That the delayed sensitivity in chickens remains detectable by one of the in vitro tests of cell-mediated immunity, with addition of another antigen that does not itself induce delayed sensitivity, has not been tested in chickens. But in vivo tests for delayed sensitivity indicated that in addition to the humoral response of BCG sensitized chickens, skin sensitivity to B-24 and in vitro leucocyte migration inhibition can be detected simultaneously. The in vivo assays for cell-mediated immunity were used to corroborate in vitro tests. The results of in vivo tests for cell-mediated sensitivity indicated that chickens in both CFA and BCG groups had detectable skin reactions with OT; however, 3 of the 8 CFA sensitized and 2 of the 8 BCG sensitized chickens had no detectable skin reactions to OT. Band-24 caused a delayed skin reaction in all of the BCG inoculated chickens and in 5 of the 8 CFA inoculated chickens. The significance of a negative skin test has long caused difficulty in diagnosis of sensitivity

and pointed to the need for additional refinements in both antigenic preparations as well as development of corroborative *in vitro* assays. The possibility that anergy or immunologic tolerance rather than true nonsensitivity is the cause of negative skin reactions cannot be ignored. In man, patients considered anergic on the basis of negative skin tests and positive bacteriologic isolation responded by migration inhibition of their lymphocytes in the MIF test (UN-WHO, 1973). To eliminate the possibility that negative skin tests indicated chickens were not inoculated, the identification of lesions and/or acid fast organisms in the membranes and/or organs of the abdominal cavity was performed. All CFA injected chickens were positive for these pathologic criteria, including those chickens with negative skin reactions. If the cell-mediated mechanism does not indicate sensitivity, the presence of precipitating antibody can indicate contact with the organism. All CFA chickens had serum antibodies to the *Mycobacteria* derived antigenic preparation PPD. All BCG inoculated chickens had precipitating antibody to PPD and only one had no lesions or acid fast organisms.

The value of diagnosis on the basis of skin tests alone is limited. The skin test is useful to screen for immunologic sensitivity, but does not differentiate present infection from previous ones. Bacteriologic isolation only can determine whether organisms are present, and the anatomic

location of the infection often precludes accessibility of samples or biopsies. A positive skin test cannot differentiate infection from disease. Because of the value of the skin test in screening for present and past infections it is of practical importance to refine antigenic preparations for use in differentiating between infections by similar organisms. While there are many shared antigens, there is a need to isolate and purify ones that are distinct and can be used to diagnose infections due to organisms with differing pathogenicity and public health significance.

The sensitivity of A-antigen in detecting MIF production in Experiment II indicated that cell-mediated immunity is stimulated by MDV but does not necessarily indicate that it is protective. Only additional studies utilizing A-antigen prepared from HVT would aid in determining whether the cell-mediated immunity is stimulated by HVT vaccination and whether this is necessary for protection against MD. The absence of lesions in MDV infected chickens was not surprising due to the reported age related resistance of chickens to MD. Antibody determinations as well as skin reactions indicated that exposure, in the absence of disease, had been effected.

The GVH assay was used in Experiment III as an additional measure of T cell function. It did not require sensitization of donors and could be used as an additional

test for T cell function in vivo. The predicted results were greater splenomegaly in embryos inoculated with leucocytes from intact and bursectomized donors than with leucocytes from thymectomized donors. The greater splenomegaly in embryos inoculated with leucocytes from thymectomized donors was unexpected. That the thymectomized group had MD, which has been reported to induce splenomegaly in embryos receiving whole blood from MDV infected chickens, was discounted because all donors were negative for fluorescent antibody to MDV at the time of the GVH assay, and throughout the duration of the experiment. The other cause for disproportionate splenomegaly, more lymphocytes in the inoculum from thymectomized donors, was discounted. Mononuclear leucocyte counts of the inoculum were made without knowledge of the donors surgical group, and adjusted to contain one million viable leucocytes. There is a possibility that the mononuclear leucocytes of blood processed from intact and bursectomized donors contained a disproportionate number of monocytes and fewer lymphocytes than the thymectomized donors. Since the GVH is lymphocyte dependent, this would have accounted for the unexpected results. However, there are no reports in the literature to support this.

Because the two sublines of line 15 used to determine embryos splenomegaly were randomly assigned to the donor chickens, it was possible that one subline had greater splenomegaly regardless of inoculum and a disproportionate

number of this subline had been inoculated with leucocytes from thymectomized donors. Therefore, the experiment was repeated with modifications. First, because the bursectomized and intact groups had given similar responses as predicted, only six each of intact and thymectomized donors were used again. This was a savings in time, embryos, and work. Second, to identify whether the two sublines were detectably different in splenomegaly from the same donor, the two sublines one and four, were identified and equal numbers of each were used for each donor. The analysis of results indicated that embryos of subline one had greater splenomegaly than those of subline four inoculated with cells from the same donor. This greater splenomegaly resulted from leucocytes of both intact and thymectomized chickens. Nonetheless, the GVH reaction by embryos inoculated with leucocytes from thymectomized donors was greater than by intact and significant at alpha 0.005.

That the leucocytes with GVH potential may not be entirely thymus-dependent has been postulated by Warner and Szenberg (1962) who reported GVH reactivity by leucocytes from adult chickens with thymic damage as a result of high doses of nortestosterone during embryonation. Sheriden and his associates (1969) reported an increase in splenomegaly by leucocytes of thymectomized donors if non-B-locus histoincompatability with recipient embryos was present, but no increase if B-locus histoincompatability existed.

They further reported that regardless of thymectomy or bursectomy that donors whose cells had strong GVH reactivity were short lived. The data here reported does not confirm the short lived observation. But the rearing of chickens, with like surgical treatments together in isolators rather than mixing all chickens and rearing them on the floor, may have given selective advantage for survival.

It cannot be excluded, according to the view expressed by Sheriden and his associates (1969), that the accentuated splenomegaly induced by leucocytes from thymectomized donors was a selective reaction to minor histoincompatabilities and this exceeded the splenomegaly by leucocytes of bursectomized and intact chickens which was to major B-locus histoincompatability. This cannot, however, be substantiated from the data in this report.

Another alternative explanation for the lack of decreased GVH reaction by leucocytes of thymectomized donors may relate to the source and preparation of leucocytes. With the exception of the report by Szenberg and Shortman (1966), leucocytes used in GVH reactions have been inoculated as part of whole blood or as spleen cell suspensions. The use of whole blood, constant volume with no adjustment for the number of mononuclear leucocytes inoculated, is questionable. Cooper et al. (1966a) reported decreased splenomegaly by blood of thymectomized x-irradiated donors and at the same time reported a significant decrease in the number of

lymphocytes in the blood of thymectomized donors. They attributed decreased splenomegaly to thymectomy, or loss of peripheral T-cells, rather than to the decrease in the number of lymphocytes in the inoculum. The degree of splenomegaly is leucocyte dose dependent, and the dose was not adjusted to be similar for all donor groups as in the study presented herein.

The results reported here use leucocytes devoid of detectable erythrocytes, but by no means purports to represent the same proportions or necessarily all types of leucocytes initially present in the whole blood. Even though MIF activity was, subsequent to inoculation with BCG, detected by leucocytes purified as for the GVH reaction, the possibility remains that GVH reactive leucocytes which remained after purification represented a partial or distinct population of mononuclear leucocytes present in whole blood. A parallel study using whole blood inoculum and purified leucocytes from each individual chicken, at identical doses of mononuclear leucocytes may indicate whether the populations of leucocytes reported in the literature as having GVH activity are the same as those reported in this study.

The thymic dependence for <u>in vitro</u> MIF production with B-24 was demonstrated in BCG infected chickens. Previous to sensitization, leucocytes were not inhibited in migration by B-24 as compared to no antigen, but after sensitization B-24 inhibited leucocyte migration. This was in agreement with

previously reported results obtained with BCG inoculated chickens (Fauser et al., 1973c). The thymectomized group values for migration in the absence, as compared to in the presence, of B-24, prior to and after sensitization were different from the intact and bursectomized values. Intact and bursectomized groups were inhibited only after sensitization and not before. The thymectomized group, on the basis of migration means, shows both apparent enhancement and inhibition in migration by B-24 prior to and after sensitization.

Because the effect of thymectomy is statistically significant in decreasing MIF production in sensitive chickens, this is supportive evidence for the role of the thymus in the development and/or expression of cell-mediated immunity or T cell function.

Because the total number of spot migrations analyzed was 2,688, the representation of results in abbreviated form was necessitated, but summation was made only after a significance of a given treatment was detected by the rigorous parametric analyses employed. To best represent the significant decrease in MIF production by thymectomized sensitized chickens as compared with intact and bursectomized chickens, the migration inhibition prior to sensitization as compared to after sensitization was calculated. The difference between intact and bursectomized groups as

compared to the thymectomized expressed as the percent increase in migration inhibition was ten-fold.

The antibody and skin test responses as well as pathologic reactions were as predicted in all experiments and were included as positive controls for cell-mediated immunity in addition to the MIF and GVH assays.

CONCLUSIONS

The development of a method for *in vitro* detection of cell-mediated immunity in the chicken contributes to future studies of the mechanisms controlling adaptive immunity. Further refinements in the methodology of cell culture are needed to reduce experimental variability of *in vitro* leucocyte migration. The development of an indirect MIF test may reduce some of the variability of results by assaying MIF using macrophages from guinea pig peritoneal exudates. It is not known whether the heterophils present in leucocyte cultures from chickens act as indicator cells in addition to the monocytes.

Inhibition of leucocyte migration occurred in both sensitized and control chickens when OT and crude MD antigens were used *in vitro* indicating a nonspecific inhibition. However, B-24 and partially purified A-antigen caused specific inhibition of migration of leucocytes from BCG and MD sensitized chickens, respectively, and not from control chickens. Both the crude and purified antigens elicit a delayed hypersensitivity reaction when inoculated intradermally. This indicates the need for purified antigenic preparations for use *in vitro* and *in vivo*. Whether the protection by HVT vaccine against MD is by the cell-mediated

immune response or whether HVT stimulates cell-mediated immunity is not known. The purification of A-antigen of HVT origin for detection of possible MIF from leucocytes of vaccinated chickens is recommended. There is potential contribution of results obtained in MD of chickens to the development of a successful vaccine against human herpesvirus induced tumors.

That the thymus is necessary for the development and/or expression for MIF production has been postulated, and results of experiments reported here support this. Leucocytes from neonatally thymectomized chickens produced significantly less MIF *in vitro* with B-24 than leucocytes from bursectomized or intact chickens after infection with BCG.

The *in vitro* MIF test, if proven adequately sensitive and reliable in detecting delayed sensitivity, could be used diagnostically and is easier than skin testing. Chickens need to be handled only once, initially for obtaining a blood sample, and not several times as for intradermal injection with antigen followed by inspection of results for several days thereafter. Also, the number of skin tests which can be made concurrently in the chicken is limited by availability of suitable dermal tissue, but *in vitro* samples can be assayed simultaneously with many antigen preparations.

The relationship between the capacity to develop delayed skin sensitivity and *in vitro* MIF production on the

one hand and the ability to induce a GVH reaction on the other may be thymus-dependent as predicted but possibly not by the same mechanism or to the same degree. MIF production and skin sensitivity were diminished in thymectomized chickens as compared with intact and bursectomized chickens. GVH splenomegaly in embryos inoculated with leucocytes from thymectomized nonsensitized donors was increased rather than diminished. Whether the previously reported thymus-dependency for GVH reactivity in chickens was leucocyte dose-dependent or reflected an absence or decrease of numbers of a T cell specifically capable of GVH reactivity should be further studied.

APPENDICES

Appendix 1

Nominal stainless steel hypodermic tubing specifications*

	OUTSIDE DIAMETER	
GAUGE	inches	mm
33	.008	.20
32	.009	.23
31	.010	.25
30	.012	.31
29	.013	.33
28	.014	.36
27	.016	.41
26	.018	.45
25	.020	.51
24	.022	.56
23	.025	.64
22	.028	.71
21	.032	.81
20	.0355	.90
19	.0425	1.08
18	.050	1.27
17	.059	1.48
16	.065	1.65
15	.072	1.83
14	.083	2.11
13	.095	2.41
12	.109	2.77
11	.120	3.05
10	.134	3.40
9	.148	3.76
8	.165	4.19
7	.180	4.57
6	.203	5.15

* Becton-Dickinson
Division of Becton, Dickinson and Company
Rutherford, New Jersey 07070

Appendix 2

Formulation of Medium 199 (M199)

COMPONENTS	mg/liter	COMPONENTS	mg/liter
AMINO ACIDS			
L-Alanine	25.0	Thiamine HCl	0.010
L-Arginine HCl	70.0	DL- α - Tocopherol-phosphate (Na_2)	0.010
L-Aspartic Acid	30.0	Tween 80	5.000
L-Cysteine HCl	0.1	Vitamin A	0.100
L-Cystine	20.0		
L-Glutamic Acid	67.0		
L-Glutamine	100.0	**OTHER COMPONENTS**	
Glycine	50.0	Adenine HCl·$2H_2O$	12.10
L-Histidine HCl·H_2O	22.0	Adenosine-5'-Mono-phosphoric acid, dihydrate (AMP) (Muscle Adenylic Acid)	0.20
Hydroxy-L-proline	10.0		
L-Isoleucine	20.0		
L-Leucine	60.0		
L-Lysine HCl	70.0		
L-Methionine	15.0	Adenosine-5'-Triphos-phate, disodium, tetrahydrate (ATP)	1.08
L-Phenylalanine	25.0		
L-Proline	40.0		
L-Serine	25.0	Deoxyribose	0.50
L-Threonine	30.0	Dextrose	1,000.00
L-Trypotophan	10.0	Glutathione (Reduced)	0.05
L-Tyrosine	40.0	Guanine HCl·H_2O	0.33
L-Valine	25.0	Hypoxanthine	0.30
		Phenol Red	20.00
VITAMINS		Ribose	0.50
P-Aminobenzoic Acid	0.050	Sodium Acetate·$3H_2O$	83.00
Ascorbic Acid	0.050	Thymine	0.30
D-Biotin	0.010	Uracil	0.30
Calciferol	0.100	Xanthine	0.34
D-Ca-Pantothenate	0.010		
Cholesterol	0.200		
Choline Chloride	0.500	**INORGANIC SALTS**	
Folic Acid	0.010	$CaCl_2 \cdot H_2O$	186.0
i-Inositol	0.050	$Fe(NO_3)3.9H_2O$	0.7
Menadione	0.010	KCl	400.0
Nicotinamide	0.025	KH_2PO_4	60.0
Nicotinic Acid	0.025	$MgSO_4 \cdot 7H_2O$	200.0
Pyridoxal HCl	0.025	NaCl	8,000.0
Pyriodoxine HIC	0.025	$NaHCO_3$	1,400.0
Riboflavin	0.010	$Na_2HPO_4 \cdot 7H_2O$	90.0

Appendix 3

Formulation of Ham's F-10 Medium (F-10)

COMPONENTS	mg/liter	COMPONENTS	mg/liter
AMINO ACIDS			
L-Alnine	8.91	Biotin	0.024
L-Arginine HCl	211.00	Choline Chloride	0.698
L-Asparagine	---	Folic Acid	1.320
L-Asparagine·H_2O	15.00	i-Inositol	0.541
L-Aspartic Acid	13.30	Niacinamide	0.615
L-Cysteine·HCl	35.12	D-Ca Pantothenate	0.715
L-Glutamine	146.20	Pyridoxine HCl	0.206
L-Glutamic Acid	14.70	Riboflavin	0.376
Glycine	7.51	Thiamine HCl	1.010
L-Histidine HCl·H_2O	21.00	Vitamin B_{12}	1.360
L-Isoleucine	2.60		
L-Leucine	13.10	**INORGANIC SALTS**	
L-Lysine HCl	29.30	$CaCl_2 \cdot 2H_2O$	44.10
L-Methionine	4.48	$CuSo_4 \cdot 5H_2O$	0.0025
L-Pheynlaline	4.96	$FeSo_4 \cdot 7H_2O$	0.83
L-Proline	11.50	KCl	285.00
L-Serine	10.50	KH_2PO_4	83.00
L-Threonine	3.57	$MgCl_2 \cdot 6H_2O$	---
L-Tryptophan	0.60	$MgSo_4 \cdot 7H_2O$	152.80
L-Tyrosine	1.81	NaCl	7,400.00
L-Valine	3.50	$NaHCO_3$	1,200.00
		$Na_2HPO_4 \cdot 7H_2O$	290.00
OTHER COMPONENTS		$ZnSo_4 \cdot 7H_2O$	0.028
Glucose	1,100.0		
Hypoxanthine	4.08		
Linoleic Acid	---		
Lipoic Acid	0.2		
Phenol Red	1.2		
Putrescine	---		
Sodium Pyruvate	110.0		
Thymidine	0.727		

Appendix 4

Suggested operation of Jamesway incubator

DAYS	TEMP. IN DEGREES FAHRENHEIT (°F)	WET BULB IN °F	EXHAUST
0	100	88-90	Closed
1	100	88	Closed
2	100	88	Closed
3	100	88-86	1/8 " Open
4	100	86	1/8 " Open
5	100	86	1/8 " Open
6	100	86	1/8 " Open
7	100	86	1/8 " Open
8	100	86	1/8 " Open
9	100	86	1/8 " Open
10	100	86	1/8 " Open
11	100	86	1/8 " Open
12	100	86	1/8 " Open
13	100	86	1/8 " Open
14	99	86	1/4 " Open
15	99	86	1/4 " Open
16	99	86	1/4 " Open
17	99	86	1/4 " Open
18	99	86	1/4 " Open
19	99	86	1/4 " Open
20	98½	88-92	1/2 " Open or more
21	98½	88-92	as required for humidity and hold temperature

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